THE SULFUR DISTRIBUTION OF PAPAIN AND RELATED STUDIES

by

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A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biological Chemistry

University of Utah
December, 1960

This Thesis for the Ph.D. Degree

by

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December, 1960

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ACKNOWLEDGEMENTS

The author is sincerely grateful to Dr. Emil L. Smith for his guidance and encouragement during the course of this research. His interest served as a constant stimulus throughout this work.

He would also like to express his appreciation to Drs. Albert Light, Robert L. Hill, and Joe R. Kimmel, for many helpful discussions.

This research was carried out during the tenure of a Pre-doctoral Fellowship from the Department of Biological Chemistry, the appointment being under the U. S. Public Health Service Training Grant. This Fellowship is gratefully acknowledged.

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CHAPTER I.

GENERAL INTRODUCTION

After the crystallization of urease by Sumner in 1926 (1), the isolation of a series of crystalline enzymes by Northrop and co-workers (2), and the rigorous identification of these as specific proteins devoid of a non-protein component, much effort has been expended to provide a satisfactory explanation for the catalytic function of proteins in terms of structural organic chemistry, reaction mechanisms and thermodynamics.

Although a number of enzymes have been subjected to intensive study both by physical and chemical approaches, the accumulation of information has not been sufficient in any one case to date to provide an unequivocal picture of the catalytic site of an enzyme, the mechanism of its action and the role of the molecule as a whole.

It is noteworthy that among the comparatively few proteins which have been subjected to extensive structural and kinetic studies, proteolytic enzymes represent the largest group. Consideration of a representative member of this group, papain, indicates many of the reasons which prompted the choice of proteolytic enzymes as subjects for study. The study of papain offers a number of advantages:

1. Papain can be prepared in a reasonable yield.
2. Synthetic substrates needed for kinetic and specificity studies may be readily prepared.
3. It is stable over a wide range of temperature and pH.
4. It is unusually stable to denaturing agents such as alcohol, urea, or guanidine.
5. It forms a mercury derivative which is useful in purification of the enzyme (3), and is potentially extremely important in crystallographic

structural analysis of papain.

On the basis of electrophoretic (3), sedimentation (3), immunochemical (4), and end-group studies (5), papain appears to be homogeneous.

The physical molecular weight as determined by the approach to equilibrium method is 20,700 (3), in excellent agreement with the value of 20,900 as determined from the amino acid composition.

The amino acid composition of papain hydrolysates has been determined by the use of ion-exchange columns described by Moore and Stein (6). The composition of papain offers several points of interest, the most notable being the complete absence of methionine and the high content of non-polar amino acid residues. Papain contains eight moles of sulfur per mole of protein and no phosphorus (7). A survey of the work on the sulfur distribution of papain is presented in a later section.

Thompson (5) using the dinitrophenylation technique was able to show that papain consisted of only one peptide chain, the N-terminal sequence being Ileu.Pro.Glu... . In the end-group determinations, DNP-amino acids other than isoleucine, were obtained in small amounts.

Investigation of the amino acid sequence of papain has been proceeding for several years. Both tryptic and chymotryptic hydrolysates of papain have been chromatographed on ion-exchange columns, and the sequences of a large number of purified peptides determined (4,8). Oxidized papain and S-carboxymethyl papain have been used as substrates in these studies. The complete amino acid sequence of this protein should be available in the near future.

The studies of Hill and Smith (9) on the degradation of mercuripapain by leucine aminopeptidase have revealed that as many as 100-120 amino acid residues may be removed sequentially from the N-terminal end leaving an

activatable fragment. This fragment has been characterized by both physical and chemical techniques and shown clearly to arise from the papain molecule. The molecular weight of the fragment is approximately 8,000. This work conclusively shows that the 80 amino acid residues at the C-terminal end of the papain molecule must include the active center. The fragment possesses the same substrate specificity as papain and is also subject to inactivation by denaturing agents (10). The information obtained with the fragment may considerably simplify the elucidation of the nature of the active site in terms of the amino acid sequence within which it is contained as well as the three dimensional configuration of the peptide chain required for its maintenance.

A major problem which remains largely unsolved to date is that of the mechanism of activation of papain. The early work on comparatively crude preparations had shown that HCN, H_2S, cysteine and glutathione all enhanced the proteolytic activity of papain (11,12,13). It was also observed that metal-binding agents possessed an activating effect and, that their effect and that of the activators mentioned above was additive in nature (14). In 1933, Bersin and Logemann (12) pointed out that all the known activators of papain were capable of reducing disulfide bonds. They successfully predicted which substances would act as activators for papain and to the activators mentioned above they added thioglycolate, thiolacetate, and sulfite. In support of their view that the activation of papain represented a reduction, Bersin and Logemann (12) showed that papain could be activated by a biological reduction system consisting of succinic dehydrogenase and succinate. Bersin (13) further showed that cystine could be reduced to cysteine by exposure to ultraviolet light and that papain could be activated by a similar treatment. It was known, at that time, that papain could be

reversibly inactivated by iodine and hydrogen peroxide and irreversibly by iodoacetic acid. These facts were in accord with the theory of Bersin and Logemann (12) since the first two agents were known to oxidize cysteine to cystine and could, therefore, be regarded as acting in the reverse direction to the postulated activation, while the effect of iodoacetate could easily be accounted for on the basis of known reactions between cysteine and halogenated acetic acid derivatives. On the basis of the above evidence, Bersin proposed that native papain existed as a dimer linked by an intermolecular disulfide bond and that activation could be represented as follows:

$$\underset{\text{Inactive}}{\text{PaSSPa}} \xrightleftharpoons[\text{oxidation}]{\text{reduction}} \underset{\text{Active}}{2\ \text{PaSH}}$$

The observations made on the crude preparations were fully confirmed by Balls and Lineweaver (7) and Kimmel and Smith (14) using crystalline papain. However, several difficulties prevent the unqualified acceptance of the activation mechanism proposed by Bersin. Firstly, papain is a monomer in the pH range and in the dilute solutions which are used for activation (4). This rules out the existence of an intermolecular disulfide bridge. Further, activated papain has only one reactive thiol group, as determined with p-mercuribenzoate or iodoacetic acid (15). If an intramolecular disulfide bond is cleaved in the activation process, the appearance of two moles of -SH per mole of the active enzyme would be expected. Thus, the simple picture of the activation of papain as a reductive cleavage of a disulfide bond is not adequate to explain the available experimental information.

Recently, the ion-exchange technique of Dintzis (16) was modified for the preparation of active reduced papain. The technique consists in the binding of thioglycolate to a strong anion exchanger and passing the enzyme

through a column containing a bed of thioglycolate resin together with a series of additional beds of ion-exchange resins introduced to buffer the solution and also to remove electrolytes so that an ion-free reduced enzyme solution is obtained (17). The column-reduced enzyme exhibits activity in the absence of activators but this activity is slowly lost on exposure to atmospheric oxygen (17). The addition of cysteine and Versene enhances the activity and stabilizes the enzyme (15). The important conclusion which may be drawn from this work is that the presence of activators is not required for the enzymic catalysis and hence these agents do not act as coenzymes.

As mentioned above, chemical evidence for the involvement of a thiol group in the active site of papain has been available for a long time (4). Papain is inhibited by all those reagents which react with thiol groups, e.g., heavy metal ions, mercurials, oxidizing agents, and alkylating agents.

Finkle and Smith (15) have shown that native papain, activated by passage through the reducing column, has somewhat less than one thiol group as determined by the spectrophotometric assay of Boyer (18) with p-mercuribenzoate, or by reaction with iodoacetate. As judged from the rate of the reaction, this thiol group is endowed with a higher reactivity towards the mercurial than the thiol groups of other proteins which have been examined by this method (15). It may be noted at this point that Balls and Lineweaver (7) showed that the active group of papain does not react with nitroprusside or porphyrindin. Finkle and Smith (15) have also found that the activity of papain appears to be linearly related to the degree of reactivity with p-mercuribenzoate. Direct chemical evidence for the involvement of a thiol group was provided by the isolation of S-carboxymethylcysteine from acid hydrolysates of the iodoacetate inhibited

enzyme (15).

Much work has been done on the kinetics of hydrolysis by papain of several synthetic substrates (19,20,21). For the substrates studied, the Michaelis-Menten formulation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_o} P + E$$

was found to be applicable on the assumption that $k_o > k_{-1}$ and, therefore, $K_m = k_o/k_1$, and hence $k_1 = k_o/K_m$. It was possible, therefore, by determining K_m and k_o, to calculate the value of k_1, the velocity constant for the formation of the enzyme-substrate complex. k_1 values have been determined over a pH range 4 to 9 at three temperatures for the hydrolysis of benzoyl-L-argininamide (BAA), and at two temperatures for each of carbobenzoxy-L-histidinamide (CHA), benzoyl glycinamide (BGA), and benzoyl-L-arginine ethyl ester (BAEE) (19,20,21). The studies with these substrates, as well as with other substrates not mentioned above, demonstrated that k_1 is strongly pH-dependent and that with most substrates the plot of k_1 vs. pH gives a bell-shaped curve with a maximum near pH 6. It was suggested that these data may be explained by assuming that the descending portions of the curves represent the titration of groups on the enzyme which are essential for the k_1 step (21). If the data are fitted with theoretical titration curves (21,22) the acidic portion of the k_1 vs. pH curve has an apparent pK' value of 4.3 (21) which is essentially temperature-insensitive. This pK' value and the lack of a temperature effect are consistent with an ionized carboxyl group. It may be emphasized here that the same k_1 vs. pH curve is found with a neutral substrate such as BGA (20) as well as with cationic substrates such as CHA or BAA (20,19). It is unlikely, therefore, that the carboxylate ion is involved in an electrostatic interaction with

the side-chain of the substrate (22). The alkaline portion of the k_1 *vs.* pH curve has an apparent pK' of 8.0-8.2 at 38° and this value is markedly influenced by temperature, the heat of ionization being approximately 5,000-6,000 calories per mole (20,21). It has been suggested that this represents the ionization of an -SH group. The calculated pK' value is much too low for ϵ-ammonium groups or phenolic -OH groups, and much too high for an imidazole group. Papain possesses only one α-amino group (5), and the participation of this group in the "active site" is ruled out on the basis of degradative studies with leucine aminopeptidase which are discussed above.

The picture of the "active site" of papain which emerges from the kinetic studies indicates the participation of both an ionized carboxyl and an un-ionized sulfhydryl in the rate-limiting step governed by k_1. The substrate specificity of papain has been the subject of a number of investigations (7,14,23). Papain shows optical specificity for the L-amino acids. Its substrate specificity is very broad. The most sensitive known substrates are those which have a strongly cationic group in the side chain (e.g., and arginyl or lysyl residue). Papain can hydrolyze synthetic substrates which possess ester or thiol ester bonds (14,24) as well as those with amide or peptide bonds. In addition to these hydrolytic reactions, papain also catalyzes synthetic and transfer reactions (23,25,26). In view of the known substrate specificity of papain and the fact that a thiol group is essential for the catalytic activity of the papain molecule, it was proposed (17) that papain functions through the formation of a thiol ester intermediate of the form $R\text{-}\overset{\overset{O}{\|}}{C}\text{-}S\text{-}E$. Since the overall rate of hydrolysis of benzoylarginine amide and benzoylarginine ethyl ester is approximately the same - a common type of intermediate for both these substrates must be postulated on thermodynamic grounds (17).

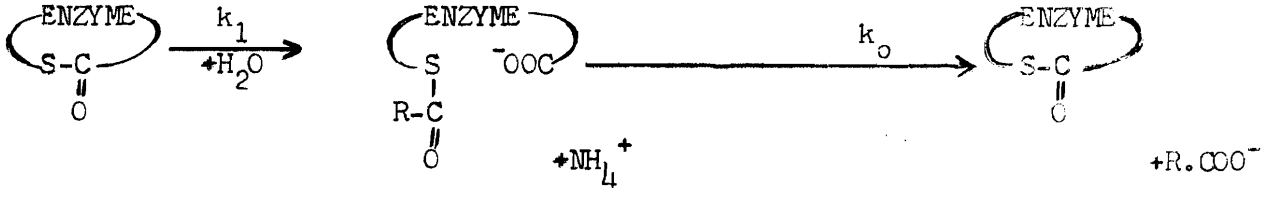
Smith (22) has recently proposed that the reactive group at the active site of papain is in fact a thiol ester (or its equivalent in terms of bond energy), formed and maintained by the folding energy of the protein. The evidence in support of this hypothesis has been presented in detail by Smith (22) and the main points may be briefly summarized as follows:

1. The free energy of hydrolysis of the susceptible bond in the amide and ester substrates of papain is far too low to permit formation of an intermediate thiol ester from a thiol group in sufficient concentration or rapidly enough to account for the observed rate of substrate hydrolysis or the efficient transferase action of papain.

2. The reactivity of the essential thiol group in papain towards p-mercuribenzoate is greater than that found with thiol groups of proteins such as β-lactoglobulin (18) or lactic dehydrogenase (27).

3. The active group of papain does not react with nitroprusside or porphyrindin (7).

4. Kinetic studies with papain indicate the involvement of an un-ionized sulfhydryl group and an ionized carboxyl group in the active site. The proposed reaction mechanism may be represented as follows:



This hypothesis provides a satisfactory explanation for most of the available information on the enzymic and chemical properties of papain.

The elucidation of the structure and the mechanism of action of papain will add greatly to the understanding of other sulfhydryl enzymes and will represent a major advance towards the solution of the general problems associated with the study of the mechanism of enzyme action.

The investigations on papain discussed above posed two major problems. Firstly, only 6 of the 8 sulfur atoms in papain were identified as half-cystine residues, the nature of the two "missing" sulfur atoms was not determined (see Chapter II Section B). Secondly, direct physico-chemical evidence for the nature of the postulated energy-rich bond at the active site of papain was lacking.

The material presented in this Thesis represents a re-investigation of the sulfur distribution of papain. The approach to the first of the problems mentioned above consisted in the application to papain of a number of well-established methods for the determination of thiol and disulfide groups in proteins. In addition, a new method for the determination of the half-cystine plus cysteine content of proteins was developed in the course of this work.

An attempt was also made to obtain evidence for the existence of the postulated thiol ester bond in active papain by employing the technique of difference spectroscopy in the ultraviolet region.

The reasoning upon which this approach was based was as follows:

1. Noda et al. (28) investigated the kinetics of hydrolysis of thiol esters in dilute aqueous media. Their study indicated that ultraviolet spectroscopy offered an accurate and sensitive means of studying both the rate and extent of thiol ester breakdown. Noda et al. (28) determined the rates of hydrolysis by following the decrease in the thiol ester absorption peak at 231 mμ. At this wavelength, the molar extinction coefficient of aliphatic thiol esters is in the range 4,000-4,500 (28). It should be possible, therefore, to demonstrate the formation of a thiol ester or its breakdown within a protein molecule by difference spectroscopy.

2. Papain is known to be readily and irreversibly inactivated by acid. As

mentioned above, the thiol ester in the active site of papain was postulated to be maintained by the folding energy of the protein. It is, therefore, reasonable to assume that acid denaturation would result in a disruption of the active site and breakdown of the thiol ester.

3. The activation of papain by cyanide could be studied by difference spectroscopy since freshly prepared cyanide solutions have a negligible absorption in the wavelength range 220-360 mμ.

4. The reaction of hydroxylamine with thiol esters, yielding hydroxamic acids, can be studied by following the disappearance of the thiol ester absorption peak at 231 mμ. Since hydroxylamine has a very low absorption in the wavelength range 220-360 mμ, this reaction can also be followed by difference spectroscopy.

Early in this study, it was observed that the ultraviolet spectrum of proteins in the wavelength range 230-235 mμ was very sensitive to conformational changes brought about by denaturation or peptide bond cleavage. The spectral changes observed were far greater than could be expected to result from thiol ester hydrolysis.

This finding made it impossible to use ultraviolet difference spectroscopy as a means of demonstrating the presence or absence of a thiol ester in papain.

Since the changes in the 230-235 mμ region reflected even very small changes in native protein structure, it was felt that a further investigation of this phenomenon would be of value. The study was, therefore, extended to include a number of proteins under a variety of conditions, amino acids, and poly-L-glutamic acid. An attempt was made to identify the chromophores in the protein which were responsible for the observed changes.

The first part of this Thesis deals with the studies on the sulfur distribution of papain while the second part is concerned with investigations employing ultraviolet difference spectroscopy.

CHAPTER II.

A. METHODS FOR THE ESTIMATION OF SULFUR IN PROTEINS - A LITERATURE SURVEY.

Among the chemically active groups of proteins, the -SH groups possess the broadest reactivity. With few exceptions, reagents used for the identification or determination of amino groups, aliphatic and aromatic hydroxyl groups, imidazole and guanidino side-chains, appear to react readily first with thiol groups. The high reactivity of the -SH groups makes it possible to use a wide variety of reagents for their quantitative determination. Unfortunately, relatively few of these reagents are absolutely specific.

Since the determination of disulfide groups, in general, involves their conversion to thiol groups or their derivatives, the above remarks apply also to the estimation of the disulfide group.

The chemistry of sulfur in proteins has been the subject of a vast amount of research and many hundreds of publications have appeared in recent years. This field has been extensively reviewed a number of times in 1959 (29-32). In the present discussion of the literature, emphasis will be placed on the methods actually employed in the present investigation of the sulfur distribution of papain. The majority of the reagents reacting with sulfhydryl groups fall into one of the following three classes:

1. Oxidizing agents, e.g., porphyrindin, sodium tetrathionate, p-iodoso-benzoate, iodine.

2. Mercaptide forming agents, e.g., p-chloromercuribenzoate, p-chloro-mercuribenzene sulfonate, silver ion, mercuric ion, phenylmercuric hydroxide, methyl mercuric iodide.

3. Alkylating agents, e.g., bromoacetic acid, iodoacetic acid, iodoacetamide.

The reagents at present most widely used for the rapid quantitative determinations of protein sulfhydryl groups are the mercaptide-forming agents and

also a reagent which does not really come within the scope of the three classes listed above, namely, N-ethylmaleimide.

The use of N-ethylmaleimide as a specific reagent for sulfhydryl groups takes advantage of the high avidity with which sulfur compounds add to the ethylene bond of maleyl compounds (33). Friedmann (34) observed changes in the absorption spectrum of N-ethylmaleimide on reaction with glutathione. These were further investigated by Gregory (35). Gregory followed the disappearance of the N-ethylmaleimide absorption peak at 302 mμ, which takes place on combination with mercaptans, as a measure of the rate and extent of the reaction. Alexander (36) and Rouser and Roberts (37) defined the conditions under which N-ethylmaleimide could be used routinely as a reagent for protein sulfhydryl groups. The method has a number of limitations, the most serious being the extreme slowness of the reaction at acid pH values and the anion-catalyzed decomposition of the reagent at alkaline pH values. Comparatively, a large quantity of material is required for an accurate determination.

In a recent paper, Smyth, Nagamatsu and Fruton (38) have reported that N-ethylmaleimide reacts with the amino group of peptides and with imidazole and histidine under conditions similar to those used for the quantitative determination of thiols with this reagent. With the amino group, and with imidazole, an N-acylation reaction appears to occur, followed in the case of imidazole by a catalytic polymerization of N-ethylmaleimide. In the case of cysteine, Smyth et al. (38) have shown in agreement with previous observations that the reaction proceeds through the addition of the thiol to the olefinic bond of N-ethylmaleimide. It may be noted that the concentration of the compounds used in this study was in the range 0.02-0.2M.

These results suggest the need for caution in the use of N-ethylmaleimide as a specific reagent for thiol groups.

It should be emphasized, however, that the use of excess N-ethylmaleimide in the determination of the -SH content of ovalbumin (36), bovine serum albumin (37), microsomal cytochrome reductase (39), and hemoglobin (40), has led to results consistent with those obtained by other methods.

The principal mercaptide-forming agent used in protein -SH estimations is p-mercuribenzoate, first studied extensively by Hellerman (41). The early quantitative studies on the reaction of p-mercuribenzoate with sulfhydryl groups were made by a titration technique with the nitroprusside test as a measure of the end-point (42). The spectrophotometric method developed by Boyer (18) represented a major simplification of the determination and resulted in its widespread use. p-Mercuribenzoate is not absolutely specific for sulfhydryl groups. Benesch et al. (43) showed by equilibrium dialysis studies of hemoglobin in phosphate buffer that only -SH groups combined at low mercurial concentrations but that non-specific combination with other groups occurred at higher concentrations. A similar observation was made by Madsen and Gurd (44) in the case of phosphorylase. It may also be noted here that the phenylmercury ion has been shown (45) to combine readily and stoichiometrically with carboxyl, amino, phenol and amide groups and that these combinations are easily broken.

Amperometric titrations have been widely used in recent years (30,46). The reactions involved are of the type

$$RSH + M^{+} \rightleftharpoons RSM + H^{+}$$

Much of the early work was carried out using the silver ion. The silver ion is univalent and readily reduced at a mercury or platinum electrode, further,

the silver mercaptides are highly undissociated. Kolthoff and Harris (47) described a method for the titration of some aliphatic thiols with $AgNO_3$ using the rotating platinum electrode and a supporting electrolyte consisting of ammonia and ammonium nitrate in 95 per cent alcohol. In this medium the silver ion is present as $Ag(NH_3)_2^+$ and consequently chloride does not interfere. The indicator electrode was adjusted to a sufficiently negative potential to reduce $Ag(NH_3)_2^+$ but not oxygen. Benesch and Benesch (48) used this method on a reduced scale for the estimation of thiols of biological interest.

The use of high concentration of alcohol is undesirable in titrations involving proteins. Accordingly, Kolthoff and Stricks (49) investigated the reaction of cysteine with silver nitrate in aqueous ammonia-ammonium nitrate buffer at pH 9.2. They found that satisfactory results were obtained by working at a low concentration range of the thiol, between 10^{-5} and 5×10^{-4}M.

Benesch, Lardy and Benesch (43) modified the method of Kolthoff and Stricks (49) by using aqueous tris (hydroxymethyl) aminomethane (Tris) buffer instead of the ammonia-ammonium nitrate buffer to avoid alkaline solutions. They carried out the titrations at pH 7.4 using the rotating platinum electrode and silver nitrate and were able to obtain satisfactory titrations with a number of proteins. Claims have been made by certain workers (50,51) that high titration values are obtained in cases of simple thiols such as cysteine and glutathione. There appears to be no simple explanation for these discrepancies. Lack of agreement was also found between the cysteine content of hemoglobin A and the -SH content as determined by argentimetric amperometric titration by the method of Benesch et al. (40). Cecil and McPhee (30) have suggested that these anomalously high results could be

explained by the formation of complexes by the silver mercaptides with silver ion (e.g., $(RSAg)_2Ag$). Formation of such complexes, even in the presence of ammonia, has been demonstrated in model systems by Kolthoff and Stricks (49).

The complexes formed by silver mercaptides with silver ions are comparable in stability to the mercaptides themselves. This is not the case with the mercuric ion. Here the mercaptides are considerably more stable than the mercaptide-Hg^{++} complexes.

Mercurimetric amperometric titrations using a rotated mercury pool electrode have been recently studied by Kolthoff and co-workers (52). The use of a divalent metal such as Hg^{++} makes it difficult to decide if the end-point of the titration corresponds to RSHgX, $[RS]_2Hg$ or $[RS]_2Hg_2$. Each new situation must be analyzed with the aid of polarography.

To avoid this problem, Allison and Cecil (53) have employed the univalent reagent phenylmercuric hydroxide and the dropping mercury electrode. This method has given satisfactory results in the case of hemoglobin A (53), human foetal hemoglobin (54), and insulin (55). Much of the early work on the -SH and S-S content of proteins has been based on analysis of the products of acid hydrolysis (56,57). While a number of methods may be employed for the estimation of the cystine and cysteine contents of an hydrolysate, it has been shown by many workers that both cystine and cysteine undergo considerable destruction during hydrolysis (58-62).

The general method of Toennies (63) for the oxidation of S-S linkages and -SH to cysteic acid residues was adopted by Sanger (64) for insulin. Performic acid, peracetic acid or bromine have all been used as oxidizing agents. This method has two advantages. Firstly, the cysteic acid residues

resulting from the oxidation are comparatively stable to acid hydrolysis under the usual conditions (71), and secondly, the introduction of new negative charges is of considerable help in protein structural studies. The procedure has found application in the study of the half-cystine content and the structure of ribonuclease (65-67), papain (68,69), chymotrypsinogen (70), bovine serum albumin (71), and wool (72).

Several difficulties are associated with the oxidation technique. The first is lack of specificity - methionine residues are converted to the sulfones and tryptophan is readily oxidized. Thompson (73) has demonstrated chlorination of tyrosine to occur when H_2O_2, used to prepare the performic acid, reacts with chloride to give chlorine. Hirs (65) has described a procedure whereby this chlorination may be prevented. Conditions giving a maximal yield of 90 per cent cysteic acid after performic acid oxidation of cystine and exposure of the product to 6N HCl at 110° for 20 hours have been described by Schram et al. (71). Unfortunately, this percentage recovery cannot be taken as applicable to individual proteins. For example, a cysteic acid recovery of 82 per cent was reported for bovine serum albumin (69) and 85-90 per cent for ribonuclease (65). Since cysteic acid is stable to acid hydrolysis, the problem appears to lie in incomplete oxidation. With incomplete oxidation, the following stages, intermediate between disulfide and sulfonic acid, are possible:

$$S\text{-}S \longrightarrow SO.\text{-}S \longrightarrow SO_2.S \longrightarrow 2\ \text{-}SO_2H \longrightarrow 2\text{-}SO_3H$$

These products are known to be unstable under the conditions normally used for acid hydrolysis of proteins and they disproportionate to give cystine, cysteic acid, and, depending on the composition of the protein studied, other products (74-77).

It was likely that the difficulties associated with the performic acid oxidation and related methods would be avoided by the use of reductive methods.

The reduction of disulfide bonds in proteins was first examined by Hopkins in 1925 (78), who demonstrated that a variety of reducing agents, including glutathione, cysteine and thioglycolic acid could bring about cleavage in a slightly alkaline solution. The early work on the reductive cleavage of disulfide bonds has been discussed in several excellent reviews (79,57). Fraenkel-Conrat et al. (80) examined the reductive cleavage with thioglycolate in their study on lysozyme. Mercaptoethanol in 8M urea was used for the reduction of the protein S-S groups. N-Ethylmaleimide or iodoacetamide were added to block the -SH groups formed and prevent aggregation by exchange reactions (81). The extent of alkylation was measured by determining the amide-N introduced, as calculated from the ratio of amide-N to total N in the original and modified protein. The results obtained indicated complete reduction and alkylation of the half-cystine residues in lysozyme.

After the work of Fraenkel-Conrat et al. (80), similar studies were carried out on a number of proteins, both in the presence and absence of denaturing agents. The reduction of disulfide bonds of human and bovine serum albumin and γ-globulin by thioglycolic acid under a variety of conditions was studied by Katchalski, Benjamin and Gross (82). The extent of reduction was measured by titration of the -SH groups produced with methylmercuric nitrate. With urea and guanidine hydrochloride as denaturing agents, complete reduction could not be achieved. Under optimal conditions reduction of only 80-90 per cent of the disulfide bonds was attained. The work on human serum albumin was extended by Hunter and McDuffie (83), who obtained complete reduction of the disulfide bonds of this protein using

thioglycollate in the presence of sodium dodecyl sulfate. After reduction, an excess of iodoacetamide was added to block the protein sulfhydryl groups and prevent their subsequent re-oxidation. Thioglycolate was also employed in the reduction of insulin (84), ribonuclease (132) and lysozyme (86). Iodoacetate was the most commonly used alkylating agent.

The determination of the extent of reduction and alkylation has been carried out by a variety of methods. The determination of the cystine content of the protein after reduction and alkylation has been employed (80, 84). Anfinsen et al. (86) estimated the S-carboxymethylcysteine content of reduced and alkylated lysozyme and ribonuclease by dinitrophenylation of acid hydrolysates of these proteins followed by chromatographic isolation of the 2,4-dinitrophenyl S-carboxymethylcysteine. A correction factor of 45-55 per cent was applied to compensate for the destruction of the dinitrophenyl derivative during the chromatography. A direct determination of S-carboxymethylcysteine following acid hydrolysis has been commonly employed (40,89).

Berger et al. (87) using carboxymethylated poly-L-cysteine have reported that there appears to be essentially no destruction of S-carboxymethyl cysteine under the usual conditions for the acid hydrolysis of proteins, i.e., in sealed evacuated tubes with constant boiling HCl at 105° for 16 hours . This report was confirmed by Sela et al. (86). On the other hand, it has been reported by several investigators that when free S-carboxymethylcysteine is subjected to acid hydrolysis under the conditions mentioned above a 5-10 per cent conversion to cystine takes place (80,84,86). It may be noted that in the case of S-carboxymethyl hemoglobin, Cole et al. (40) have reported a decomposition of approximately 8 per cent during 22 hours of hydrolysis.

Sodium borohydride has also been employed as a reducing agent (88,89). It has been shown, however, that the reduction may be accompanied by cleavage of peptide bonds (89), and is thus unsuitable both for quantitative and structural studies.

Both from the viewpoint of structural studies and as a method for the quantitative determination of protein half-cystine plus cysteine content the reductive cleavage of disulfide bonds followed by alkylation with iodoacetate or iodoacetamide is superior to the oxidative methods.

Two points should be kept in mind. Firstly, the denaturation conditions necessary for the complete reduction of the disulfide bonds have been found to vary enormously from protein to protein. Secondly, the work of Gundlach et al. (90,91) has indicated the importance of carefully controlled conditions in determining the specificity of the alkylation step.

Much attention has been devoted recently to the nucleophilic cleavage of the disulfide bond (32,94). A reaction of the disulfide bond which has assumed major importance is the reaction with sulfite. This reaction involves cleavage of the disulfide bond with addition, leading to the formation of a thiol and a sulfonic acid group (92,93):

$$RSSR + SO_3^{=} \rightleftharpoons RS^- + RSSO_3^-$$

Kinetic studies have shown that above pH 9 the reaction is a simple bimolecular one, as shown above, but below pH 9 it is more complicated (95). $SO_3^{=}$ has been shown to be the attacking ion, HSO_3^- does not attack the disulfide bond. It is of importance to note that the equilibrium of the cleavage reaction is considerably to the left (96). In the reaction of cystine and of dithioglycolic acid with sulfite, the Keq. is of the order of 0.01 (96). The position of equilibrium in a given system is determined

by lowering the pH to 2. At this pH all of the $SO_3^=$ is present as HSO_3^- and all of the RS^- is present as RSH.

In the presence of a mercaptide forming agent, e.g., Hg^{++} or Ag^+, the reaction is pulled to the right owing to the removal of RS^- according to the scheme:

$$RSSR + SO_3^= \rightleftharpoons RS^- + RSSO_3^-$$
$$RS^- + H^+ \rightleftharpoons RSH$$
$$RSH + Ag^+ \rightleftharpoons RSAg + H^+$$

The above scheme forms the basis of the amperometric and polarographic methods for the determination of the disulfide content of proteins (30).

Essentially, all these methods involve the cleavage of the S-S bonds by sulfite in alkaline solution in presence of the mercaptide-forming agent. The -SH formed may be determined either by amperometric titration with silver nitrate and the rotating platinum electrode (97,98), or by amperometric titration with mercuric chloride and a rotated mercury pool electrode developed by Kolthoff and co-workers (99).

Carter (97) has titrated a number of proteins at 37° in 8M urea immediately following the addition of sulfite. These titrations, carried out with silver nitrate and the rotating platinum electrode, yielded values in excellent agreement with those obtained by other methods. Resnick et al. (100) have used Carter's technique to study the effect of disulfide bond cleavage on the enzymic activity of ribonuclease.

Treatment of cystine and cysteine with ammoniacal cupric sulfate has been shown to result in the conversions shown below (130):

$$RSSR + 2Cu^{++} + 2SO_3^= \rightleftharpoons 2RSSO_3^- + 2Cu^+$$
$$RSH + 2Cu^{++} + SO_3^= \rightleftharpoons RSSO_3^- + 2Cu^+ + H^+$$

These reactions are extremely rapid and appear to be highly specific. A notable advantage is that a symmetrical cleavage of a disulfide is obtained. A method based on these reactions has been developed by Swan (31) and employed for dissolving keratin.

Swan's method has been successfully applied by Pechere et al. (101) to the cleavage of disulfide bonds in trypsinogen and chymotrypsinogen. Bailey (102) has applied it to insulin. Weil and Seibles (103) have used a modification of Swan's procedure for the cleavage of the disulfide bonds in α-lactalbumin and β-lactoglobulin. In all the cases listed above, full conversion was achieved and the resulting derivatives were soluble. It is of interest to note that while 8M urea was required for the complete conversion of the cysteinyl and cystyl groups into S-sulfocysteinyl groups in the keratins, trypsinogen and chymotrypsinogen - full conversion was obtained in the case of α-lactalbumin and β-lactoglobulin in the absence of denaturing agents (103). In some cases the S-sulfocysteinyl content of the proteins was determined by S^{35} incorporation. In others, the increase in total sulfur was measured by the titrimetric Carius combustion method (104) and used as a measure of the conversion (103).

A serious disadvantage of the Swan procedure is the prolonged dialysis necessary to remove the cuprous and cupric ions. This disadvantage may be avoided by using the method of Bailey and Cole (98), in which iodosobenzoate or tetrathionate are used as the oxidants instead of the cupric ions. Another disadvantage, which is not so easily obviated, is the instability of the $RSSO_3^-$ group in alkaline solution and its destruction on acid hydrolysis.

B. PREVIOUS WORK ON THE SULFUR DISTRIBUTION OF PAPAIN.

The observations on the activation and inactivation of papain and the chemical identification of S-carboxymethyl cysteine in hydrolysates of iodoacetate-inactivated papain (4) support strongly the view that a thiol group is present in the "active site" of this enzyme. The sulfhydryl nature of papain has prompted a number of investigations on the nature of the sulfur present in this protein. Balls and Lineweaver (7) determined the sulfur content of papain to be 1.2 per cent. Subsequent determination of the sulfur content by Kimmel and Smith (14) yielded the value of 1.22 per cent. The molecular weight of papain is approximately 20,500 on the basis of the amino acid composition and physical measurements (4). On the basis of this molecular weight the percentage of sulfur determined indicates that there are eight moles of sulfur per mole of papain. Since papain contains no methionine, it was concluded that the sulfur of papain could be accounted for by the cystine and cysteine content of this protein (6). However, determination of the half-cystine content of papain as cysteic acid after performic acid oxidation (69) or bromine oxidation (68) indicated the presence of only six half-cystine residues per mole of papain. A serious discrepancy thus existed between the sulfur content of papain, 8 moles of sulfur per mole of protein, and the apparent half-cystine content of 6 residues per mole of protein. Attempts were made to detect the form in which the two missing sulfur atoms were present within the papain molecule. It may be noted at this point that analysis of native, performic acid oxidized and sodium borohydride reduced papain gave 8 moles of sulfur per mole of papain in each case. Finkle and Smith (15) could find no inorganic sulfate in papain hydrolysates thus excluding the presence of either labile sulfate esters or bound inorganic sulfate.

Kimmel and Smith (14) were unable to detect thiolhistidine and Thompson (68) could find no lanthionine in papain.

A further study of the sulfur content of papain was carried out by Finkle and Smith (15). In this study it was shown that incubation of papain with excess p-chloromercuribenzoate or p-chloromercuribenzoic acid in 70 per cent alcohol resulted in the binding of 4-6 moles of p-chloromercuribenzoate or p-chloromercuribenzene sulfonic acid per mole of papain as determined by mercury analysis. At the time this study was carried out, it was not known that p-chloromercuribenzoate could act as a disulfide cleaving agent. Also, it was thought that papain contained only one histidine residue whereas it is now known that there are two moles of histidine per mole of papain (4). The results of Finkle and Smith (15) were interpreted to mean that papain contained six sulfhydryl groups per mole of protein and that it was possible that some preparations contained four sulfhydryl groups and one disulfide. Finkle and Smith (15) also investigated the sulfhydryl content of native papain activated by passage through an ion-exchange resin column containing IRA-400 in the thioglycolate form. It was shown in this study that native papain activated in this manner contained less than one mole of sulfhydryl per mole of protein as determined by direct spectrophotometric determination of p-chloromercuribenzoate or reaction with iodoacetate. The activity of the preparation appeared to be a function of the reactive thiol content.

The work presented in the first part of this thesis deals with a reinvestigation of the sulfur distribution by a number of methods.

CHAPTER III.

THE SULFUR DISTRIBUTION OF PAPAIN

A. SULFHYDRYL CONTENT OF THE PROTEIN

The work presented in this section represents a study of the sulfhydryl content of papain under a variety of conditions. The reagents used for the -SH determinations were p-mercuribenzoate, N-ethylmaleimide and Ag^+ ion.

EXPERIMENTAL

a. Materials.

Papain and mercuripapain were prepared by the method of Kimmel and Smith (105). The papain preparations were recrystallized twice and the mercuripapain preparations recrystallized once. The C_1 values of the papain preparations used were 1.5-1.7. The C_1 of the mercuripapain was 1.4, and that of the column-reduced preparation, 1.7. Assays of proteolytic activity were carried out by the Grassman-Heyde procedure (106) with benzoyl-argininamide as substrate. Protein concentrations were determined by ultraviolet absorption. $E^{1\%}_{1\ cm}$ at 278 mμ was found to be 25.0 for papain at pH 7.0 (moisture free, ash-free basis).

p-Mercuribenzoate[1] was obtained from Sigma. It was recrystallized once by the method of Boyer (18). N-Ethylmaleimide[1] was a Fischer reagent. Reduced glutathione was obtained from Schwartz. Stock 0.1N silver nitrate solution was prepared by direct weighing of the reagent. It was stored in the dark. Urea was a Mallinckrodt AR reagent. It was recrystallized from 70 per cent aqueous alcohol at 40-50°, small crystals were obtained by rapid

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[1] The following abbreviations are used: NEM, N-ethylmaleimide; PCMB, pmercuribenzoate; di-DNP-cystine, N,N'-Bis-2:4-dinitrophenyl-L-cystine; mono-DNP-cystine, N-2,4-Dinitrophenyl-L-cystine.

cooling to -10°. Guanidine hydrochloride was prepared from guanidine carbonate and recrystallized from aqueous methanol. Sodium lauryl sulfate was a duPont Nemours analytical grade reagent.

b. Methods.

1. N-Ethylmaleimide.

The determinations involving N-ethylmaleimide were carried out by the method of Alexander (36). The rate and extent of reaction were measured by following spectrophotometrically the decrease in absorption of the N-ethylmaleimide at 300 mμ. The molar extinction coefficient of N-ethylmaleimide at 300 mμ was found to be 620. This agrees with the value given by Alexander (36). The absorbancy measurements were performed with a Beckman DU spectrophotometer and matched 1 cm cells. The reaction mixtures were 0.001M NEM and the protein concentration was in the range 1-5 x 10^{-4}M. Concentrated urea solution or sodium lauryl sulfate were used as denaturing agents in some experiments. It was found that neither of these denaturing agents had any effect upon the determination of the sulfhydryl content of reduced glutathione. A similar observation has been made by Cole et al. (40). Experiments were generally carried out in duplicate - known amounts of glutathione being added to one of each pair. The glutathione recovery was 96 per cent or better in all cases. Papain solutions were prepared freshly for each set of experiments and in no case kept longer than 24 hours at 4°. No difference in the -SH titer could be observed when the protein was dissolved directly in the buffered N-ethylmaleimide reagent.

2. p-Mercuribenzoate.

The spectrophotometric method of Boyer (18) was employed for these determinations. This procedure with p-mercuribenzoate is based on the increase in absorbancy in the 250 mμ region accompanying mercaptide formation.

The molar change in absorbancy ($\Delta\epsilon_M$) at 255 mμ per mole of papain sulfhydryl was found to be 8.2×10^3, slightly lower than the value of 8.5×10^3 reported by Finkle and Smith (15). Because of the difficulty in ensuring that the p-mercuribenzoate is complete dissolved, the stock solutions were centrifuged and concentration determined by measurement of the absorption both at 232 mμ at pH 7 (ϵ_M 1.69×10^4) and 234 mμ at pH 4.6 (ϵ_M 1.74×10^4) following dilution of an appropriate aliquot.

Molar ratios of PCMB to papain ranging from 2 to 10 were used; no dependence of the thiol titer on the amount of PCMB present in this range could be detected. Reduced glutathione was used as a control in a number of the experiments and recoveries of 97-100 per cent were obtained routinely. No difference in the sulfhydryl titer was observed whether the protein was added to the reaction mixture already containing the mercurial or whether the order was reversed.

3. Determination of Sulfhydryl Groups by Amperometric Titration.

The apparatus and methods used are described in Chapter II, Section D entitled "Determination of Sulfhydryl and Disulfide Groups by Amperometric Titration".

c. RESULTS

1. Sulfhydryl Content of Native Unactivated Papain

(i) N-Ethylmaleimide

Native papain yielded titers varying from 0.20-0.45 moles -SH/mole protein. The titer was dependent upon the preparation used. In the presence of sodium lauryl sulfate only trace amounts of sulfhydryl could be titrated. No sulfhydryl could be detected after urea or acid denaturation, even in experiments in which N-ethylmaleimide and Versene were added together with the denaturing agent to the protein solution. No sulfhydryl could be

detected in either native or denatured mercuripapain. The conditions used and the results obtained are detailed in Table I.

(ii) p-Mercuribenzoate.

Determinations with this reagent were carried out under a variety of conditions. As observed above for N-ethylmaleimide, addition of denaturing agents did not increase the -SH titer. Mercuripapain, once again, did not give a significant -SH titer either in the native or denatured state. The results and details of the conditions used are presented in Table I.

(iii) Amperometric Titrations.

Native papain, papain denatured with sodium lauryl sulfate or 8M urea gave -SH titers of 0.1-0.2 μmoles -SH/mole papain, as determined by the argentimetric amperometric titration of Benesch et al. (43). Acid denatured papain titrated in 8M urea at pH 7.4 gave no -SH titer. The results are presented in Table V.

It may be noted, that in the experiments carried out by the "excess reagent" method (see Chapter I, Section D-b(2)), incubation of the protein with molar ratios of $AgNO_3$/protein ranging from 2.5 to 20, in 8M urea pH 7.4, for varying periods of time, did not affect the -SH titer. These are the conditions under which anomalous binding of silver to hemoglobin was observed by Cole et al. (40). The fact that papain gives the same thiol titer under the various conditions of denaturation and Ag^+ concentration used indicates the absence of artifacts due to non-specific binding of silver ions.

TABLE I.

REACTION OF PAPAIN WITH NEM AND PCMB.

0.1M phosphate buffer at pH 7.0 was used for the determinations with NEM.

0.33M acetate buffer at pH 4.6 was used for the determinations with PCMB.

Protein Preparation	-SH Reagent	Denaturing Agent	Number of Determinations*	-SH Titer
Unactivated Papain	NEM	None	4	0.32 ± 0.13
		2.4% sodium lauryl sulfate	2	0.10 ± 0.05
		Acid denaturation at pH 1.2 for 10 minutes	2	0
		1.6% sodium lauryl sulfate in presence of 0.01M Versene	2	0.10 ± 0.05
Mercuripapain		None	2	0
		2.4% sodium lauryl sulfate	2	0
Unactivated Papain	PCMB	None	3	0.24 ± 0.10
		7.6M urea	2	0.19 ± 0.02
		7.6M urea, 1.5 hours at 70° in presence of PCMB	2	0.17 ± 0.02
		1.0% sodium lauryl sulfate	2	0.32 ± 0.03
Mercuripapain		None	1	0.04
		7.6M urea	2	0.005 ± 0.005

* Each replicate determination was carried out on a different unactivated papain or mercuripapain preparation. The value given for the -SH titer represents the mean of the values obtained with different preparations.

(iv) Summary of the Results.

The results obtained with NEM, PCMB and amperometric titrations with silver nitrate indicate that native, unactivated papain contains less than 0.5 mole -SH/mole of protein. The exact titer is dependent on the individual preparation examined. Denaturation of papain, regardless of the agent and procedure used, does not increase the -SH titer. The results obtained by the three different methods employed for the determination of -SH are in excellent agreement on this point. This clearly indicates the absence of "masked" thiol groups in papain.

No sulfhydryl could be detected in mercuripapain either in presence or absence of denaturing agents.

2. Sulfhydryl Content of Column-Activated Papain.

(i) N-Ethylmaleimide.

Titration of column-reduced papain with N-ethylmaleimide within 24 hours of passage through the column led to a value of approximately one mole of sulfhydryl per mole of papain. Prolonged storage, even at 4°, led to a decrease in the titer to about 0.5 moles -SH per mole of the enzyme. No increase in the sulfhydryl titer could be brought about by denaturation. The decrease in the -SH titer of papain on storage is analogous to that reported by Simpson and Saroff (107) for the sulfhydryl group of bovine serum albumin.

(ii) p-Mercuribenzoate.

In agreement with the observations of Finkle and Smith (15), it was found that freshly column-reduced papain reacts with 0.6-1.0 moles of p-mercuribenzoate per mole of enzyme. The results obtained with the mercurial paralleled those described above for N-ethylmaleimide. The reaction between the reactive thiol of papain and PCMB was over within 10 minutes. This

observation agrees with the findings of Finkle and Smith (15).

The results obtained with both NEM and PCMB are tabulated in Table II. Since these experiments represented a confirmation of the results of Finkle and Smith (15), only one preparation of column-reduced papain was investigated.

(iii) Summary of the Results.

Passage of papain through the 5-bed thioglycolate column (15) increases the thiol titer as measured by both the PCMB and NEM methods from 0.1-0.4 moles -SH/mole for the preparations prior to passage through the column to 0.5-1.0 moles -SH/mole papain for the column-reduced material.

Since denaturation of the column-reduced material does not increase the -SH titer, it would appear that no "masked" -SH groups, in the usual sense, are present.

d. Conclusions.

1. Native, unactivated papain gives a titer of 0.1-0.4 moles -SH/mole papain, as determined with PCMB, NEM or silver ion.

2. No increase in the thiol titer can be obtained by various methods of denaturation. This indicates the absence of "masked" thiol groups.

3. Column-reduced papain gives a -SH titer of 0.5-1.0 moles -SH/mole papain with NEM or PCMB. No increase in titer can be produced by denaturation.

TABLE II.

REACTION OF COLUMN-REDUCED PAPAIN WITH NEM AND PCMB.

0.1M phosphate buffer at pH 7.0 was used for the determinations with NEM.

0.33M acetate buffer at pH 4.6 was used for the determinations with PCMB.

Protein Preparation	-SH Reagent	Denaturing Agent	Number of Determinations*	-SH Titer
Column-reduced papain	NEM	12 hours at 4°	2	1.01 ± 0.10
		96 hours at 4°	1	0.47
		96 hours at 4° followed by 10 min. in 6.46M urea	1	0.49
	PCMB	Immediately after column-reduction	2	0.68 ± 0.05
		12 hours at 4°	1	0.62
		1.5 hours at 30° in 7M urea in presence of PCMB immediately after column-reduction	1	0.90
		7M urea, 92° 30 min.	1	0.79
		24 hours at 4°, followed by 10 min. in 5.3M guanidine hydrochloride	1	0.30

* Only one preparation of column-activated papain was investigated.

B. DISULFIDE INTERCHANGE REACTION

The interchange reaction between disulfides in concentrated acid solutions was first demonstrated by Sanger (108) in 1953. Sanger showed that incubation of a mixture of cystine and di-DNP-cystine [1] in concentrated hydrochloric acid led to the formation of mono-DNP-cystine [1]. Ryle and Sanger (109) investigated this reaction further using both cystine and insulin, and made the following observations:

(a) The reaction was very slow at HCl concentrations below 9N, but showed only a slight dependence on the acid concentration above this value.

(b) The interchange reaction in acid solution was strongly inhibited by thiols.

In 1957, Benesch and Benesch (110) investigated the mechanism of the disulfide interchange reaction using cystine and di-DNP-cystine in concentrated hydrochloric acid as a model system. They made the following observations:

(a) The reaction was found to be quite fast in concentrated hydrochloric acid, but the rate fell off rapidly at concentrations below 9N.

(b) The reaction was catalyzed by cystine disulfoxide, hydroperoxides, and aromatic and aliphatic sulfenyl chlorides.

(c) The reaction comes to an equilibrium when 80 per cent interchange has taken place. Cystine was present in ten times the amount of di-DNP-cystine in the reaction mixtures.

Benesch and Benesch (110) pointed out that all of the compounds which were found to be effective catalysts in the exchange reaction, give rise to sulfenium ions in strongly acid solutions. They concluded that the following mechanism could be proposed for the disulfide interchange reaction

in acid solution:

<u>Initiation:</u> $RSSR + H^+ \rightleftharpoons RS^+ + RSH$ (1)

<u>Interchange:</u> $RS^+ + R'SSR' \rightleftharpoons RSSR' + RS^+$ (2)

<u>Inhibition:</u> $RS^+ + R'SH \rightleftharpoons RSSR' + H^+$ (3)

A similar reaction in which a number of symmetrical disulfides (RSSR) reacts with 2,4-dinitrobenzenesulfenyl chloride or 4-carboxy-2-nitrobenzenesulfenyl chloride (ArSCl) in acetic acid at room temperature to give unsymmetrical disulfides has been reported by Moore and Porter (129), who proposed the following mechanism:

$$\begin{array}{ccccc} \overset{\delta+\ \delta-}{ArS\text{-}Cl} & & ArS\overset{\curvearrowright}{\text{-}}Cl^- & & ArSSR \\ & \rightleftharpoons & \uparrow & \longrightarrow & + \\ R\ddot{S}\text{-}\ddot{S}R & & [RS\text{-}SR]^+ & & RS\text{-}Cl \end{array}$$

A polar mechanism involving separation of charges in the transition state would seem to be involved, since Moore and Porter (129) have shown that less polar solvents decrease the rate of exchange.

Benesch and Benesch (110) were able to show, in agreement with the observations of Ryle and Sanger (109), that the rate of the interchange reaction was greatly decreased by thiols.

This section describes the use of the interchange reaction for the estimation of the cystine plus cysteine content of proteins. Briefly, the procedure involves the following steps: (a) Incubation of protein with an excess of di-DNP-cystine in strong hydrochloric acid at 39°, (b) withdrawal of aliquots at intervals, (c) extraction of di-DNP-cystine from these samples with ether, and (d) spectrophotometric determination of mixed disulfide remaining in the aqueous acid solution after the ether extraction.

The method has given reproducible results with many proteins. For proteins with known disulfide and sulfhydryl content, the agreement of the values obtained with the published values is excellent. It may be noted that in the studies of Benesch and Benesch (110) and Ryle and Sanger (109), either cystine or protein (in terms of its disulfide content) were in excess of the di-DNP-cystine. In the present work this situation has been reversed, a considerable excess of di-DNP-cystine compared to the cystine content of the reaction mixture being maintained.

EXPERIMENTAL

Materials and Methods

Materials - N,N'-Bis-2,4-dinitrophenyl-L-cystine (di-DNP-cystine) was prepared by the method of Porter and Sanger (111) and its purity checked chromatographically. N-2,4-Dinitrophenyl-L-cystine was prepared as described by Ryle and Sanger (109). Its molar extinction coefficient in 6N HCl is 15,000 at 357 mμ and its purity was determined chromatographically.

Cystine was an A. R. reagent obtained from Winthrop-Stearns. Cysteine-HCl A. R. was obtained from Nutritional Biochemicals Corp. and contained 89 per cent sulfhydryl by the N-ethylmaleimide and p-chloromercuribenzoate methods. Glutathione (Schwartz BioResearch, Inc.) contained 98 per cent sulfhydryl as determined by the p-chloromercuribenzoate and N-ethylmaleimide methods. Analytical reagent grade hydrochloric acid was used.

Procedure - A weighed amount of the protein preparation was dissolved in a volume of 9.6N HCl such that the concentration of protein was in the range 0.025-0.20 μmoles per ml. The solution was then divided into two equal parts. One part represented the blank. To the other part

1.2 mg di-DNP-cystine per ml [2] were added and the vials were closed tightly and placed in a water bath at $39^{\circ} \pm 0.03^{\circ}$, unless otherwise specified. Prior to immersion, the vials were wrapped with aluminum foil. Small glass vials with polyethylene covers which provided an airtight seal were used throughout. After immersion for several hours in the bath, the aluminum foil adhered tightly to the glass forming an excellent protection against light. Samples were withdrawn from the reaction mixture at the time intervals desired.

Aliquots of 1 ml were withdrawn and pipetted directly into vials containing 1.5 ml water and 3.0 ml ether. The di-DNP-cystine was then extracted once with 6 ml and three times with 3 ml of ether. The ether present in the aqueous phase was evaporated by gentle suction and the aqueous phase was transferred quantitatively to a 25 ml volumetric flask and diluted to the mark with 6N HCl.

The use of 6N HCl was necessary in cases where it was desired to study the rate of the reaction within 6-18 hours of the start of the experiment. In the case of some proteins, turbidity was observed when the aqueous phase was diluted with water or HCl at concentrations lower than 6N.

The absorbancy (A) at 357 mμ in 1 cm. cells of the diluted aqueous phase, and of the blank which had been treated in exactly the same manner, was then determined. Either a Beckman DU or a Cary Model 14 Spectrophotometer were used. The calculation is then, simply: μmoles of mono-DNP-cystine per ml reaction mixture = 25 A/15 = 1.67 A.

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[2] Under the conditions used, a saturated solution of di-DNP-cystine was found to contain 1.3 μmoles/ml. Since 1.2 mg di-DNP-cystine represents approximately 3.3 μmole the solution is saturated throughout the interchange experiment, solid di-DNP-cystine remaining at the bottom of the vial. Upon checking the di-DNP-cystine concentration at different stages of the interchange with various proteins, it was observed that in each case the interchange reaction was sufficiently slow to allow the solid di-DNP-cystine to compensate exactly for the amount of di-DNP-cystine used in the interchange reaction.

The interchange reaction was generally followed until the mono-DNP-cystine concentration in the aqueous phase did not change by more than one per cent over a period of 4 days. When approaching equilibrium, the determinations of mono-DNP-cystine were carried out in triplicate and the values obtained averaged.

Determination of Protein Content - In most cases, protein concentration was determined by using the known extinction coefficient of the protein in question in the region of 280 mμ. For maximal precision, the extinction coefficient of the protein in 6N HCl was compared with that of a neutral solution at the same concentration of protein. The correction involved was of the order of 2-5 per cent, the absorbancy being less in all cases in the 9.6N HCl as compared with the neutral solution. Once the extinction coefficient in 9.6N HCl was available, a direct determination of the protein concentration in the reaction mixture blank could be performed. It was found for all the proteins studied that when such a determination was performed within 30 minutes of dissolving the protein in 9.6N HCl, no measurable loss of tyrosine or tryptophan had taken place at room temperature. In a few cases, where values of $E^{1\%}_{1\ cm}$ were not available, the protein content was obtained by performing dry weight determinations and correcting for the moisture content of the protein.

In general, it was found that deionized proteins gave lower blank values and the use of such preparations is advised if high accuracy is desired.

Aliquots were removed from reaction mixtures containing different proteins and di-DNP-cystine at different times during the interchange reaction and after equilibrium was reached. These samples were extracted with ether as described above. Two-dimensional chromatography was performed

with the "toluene" system (112) in the first dimension and sodium citrate in the second dimension (131). Pyridine-acetic acid-water buffer at pH 6.4, was used for the electrophoresis (113) and n-butanol-acetic acid-water (200:30:75) for the chromatography in the two-dimensional electrophoresis-chromatography. Examination of the chromatograms after both of these separations revealed that the ether phase contained only di-DNP-cystine. No other DNP-derivatives or ninhydrin positive material could be detected. The aqueous phase contained no trace of di-DNP-cystine.

No breakdown of di-DNP-cystine was observed after 4 weeks at 39° or after 48 hours at 60° in 9.6N HCl. After more than a few days at 60°, some breakdown of di-DNP-cystine was observed. Since the concentration of the mono-DNP-cystine peptides present at equilibrium (as determined spectrophotometrically) remained constant over a considerable period of time, it is apparent that the mono-DNP-cystine is also stable under the conditions used. It should be emphasized that the reactions were studied with all light excluded since it is well known that these DNP derivatives are light-sensitive.

General Observations and Optimal Conditions

In agreement with the findings of Ryle and Sanger (109), it was observed that the rate of interchange in 12N HCl was essentially the same as that in 9.6N HCl.

"Aging" of the di-DNP-cystine Solution: Benesch and Benesch (110) observed that the interchange reaction proceeded at a faster rate when either or both of the participants had been allowed to stand for a long period of time in 12N HCl. They considered this effect to be due to the accumulation of RS^+ ions.

A saturated solution of di-DNP-cystine in 12N HCl was allowed to stand in the dark for 2 months. The rates of interchange obtained with this

reagent and a freshly prepared one were then compared in the case of several proteins. Although the initial rate of interchange was somewhat increased, this effect disappeared after several hours and the rate became identical to that observed with the freshly prepared reagent.

Effect of Temperature on the Rate of Interchange - It was found that when the interchange reaction was carried out at 60°, the rate of the reaction was appreciably increased. There are, however, two disadvantages to using this high temperature. First, the equilibrium concentration of mixed disulfide for a given protein concentration was approximately 10 per cent less than that obtained at 39°, thus lowering the precision of the measurements. Secondly, some destruction of di-DNP-cystine took place at 60° whereas no detectable destruction of the reagent could be observed at 39°. In view of these findings, it was considered that the disadvantage of the longer time taken to attain equilibrium at the lower temperature was offset by the advantage of greater precision.

Reaction with Thiols and Effect of Added Cysteine - The interchange reaction occurred with both cysteine and glutathione although at a very much slower rate than with either cystine or ribonuclease (Fig. 1).

When added to a disulfide, such as cystine, cysteine was found to slow down the rate of the interchange reaction considerably (Fig. 1). The same effect was observed on addition of cysteine to a protein containing only disulfide bonds, e.g., insulin. The extent of decrease in rate of the interchange depended on the relative amount of protein and cysteine. It should be noted that when sufficient time was allowed for the mixture to attain equilibrium, i.e., several weeks, the final value of mixed disulfide represented the sum of the added cysteine and the disulfide content of the protein.

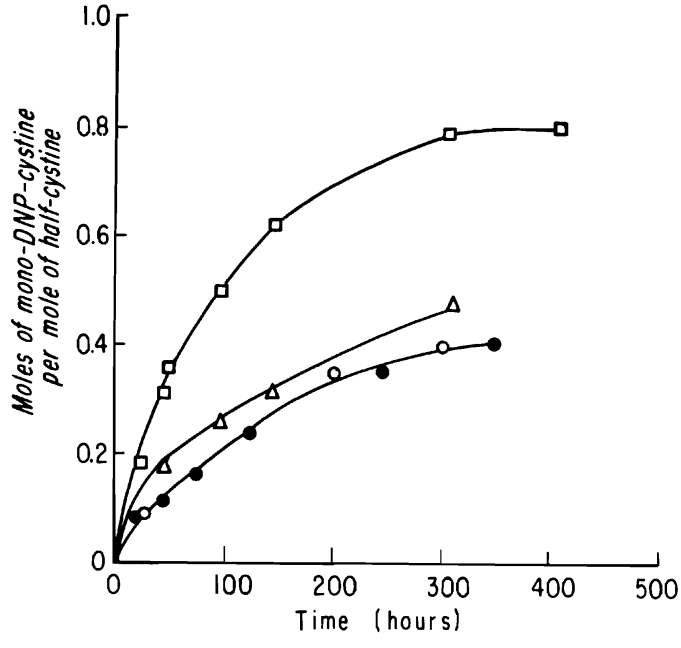

Fig. 1. Reaction of thiol and disulfide containing compounds with di-DNP-cystine in 9.6N HCl at 39°. □ Cystine (0.6 μmole/ml); ○ cysteine (1.6 μmole/ml); ● glutathione (1.6 μmole/ml); △ cystine (0.6 μmole/ml) plus cysteine (0.4 μmole/ml).

Rate and Equilibrium of the Interchange Reaction - The rate at which equilibrium was attained depended markedly on the protein under investigation. Of the proteins studied, ribonuclease was found to reach equilibrium most rapidly, within 48 hours, whereas trypsin attained equilibrium only at the end of approximately 21 days. Some data for several representative proteins are presented in Fig. 2 and 3.

In the half-cystine concentration range 0.1-3.4 μmoles per ml of reaction mixture, it was observed that as the concentration of half-cystine (i.e., protein) was increased the amount of the mixed derivative per mole of half-cystine, obtained at equilibrium was diminished (Fig. 3). This is probably due to the fact that as the absolute concentration of the mixed derivative becomes comparable with that of the di-DNP-cystine, the reverse reaction leading to the formation of symmetrical disulfide becomes important.

$$R\text{-}S\text{-}S\text{-}R' \quad + \quad R'S^{+} \rightleftharpoons R'\text{-}S\text{-}S\text{-}R' \quad + \quad RS^{+}$$

This observation was utilized for the determination of the half-cystine content of proteins. The amount of mono-DNP-cystine present at equilibrium was determined at several protein concentrations for each protein and the ratio of μmole mono-DNP-cystine per μmole of protein was calculated for each concentration of protein. The values thus obtained were plotted against the protein concentration and the line, calculated by the method of least squares, through these points was extrapolated to zero protein concentration. The intercept represents the limiting number of mono-DNP-cystine residues and hence the total cysteine and half-cystine content of the protein. Several such representative plots are shown in Fig. 4.

Table III gives for a number of proteins the half-cystine values obtained by the extrapolation method described above. It is evident that for these proteins the values determined by this method are in excellent agreement with the published values for the content of half-cystine plus cysteine. For the sake of brevity, only a few of the more recent literature values are cited.

The equilibrium values for the mono-DNP-cystine formed may be plotted as a function of the total cysteine plus half-cystine content of the reaction mixture. Such a plot is given in Fig. 5 for bovine serum albumin, trypsin and ribonuclease. It is evident that the points fit on a single smooth curve and, indeed, data obtained with other proteins also fit the same curve very well.

Inasmuch as the data for all proteins studied fit on the same curve, it becomes possible to determine the half-cystine content of a protein on the basis of a single determination, provided that a curve such as the one in Fig. 5 has been constructed, or simply by using the curve in Fig. 5. The most precise values are obtained when the half-cystine content of the reaction mixture is in the range of 0.5-1.5 μmoles of half-cystine per ml. It should be emphasized, however, that the value obtained on the basis of a single determination will be less reliable than that obtained by the extrapolation method.

In Table IV some results are given for several proteins. In these instances duplicate determinations of the equilibrium values for the mono-DNP-cystine formed, at a single protein concentration, were performed. The results were then calculated by interpolation on the smooth curve of Fig. 5. The data in Table IV indicate that satisfactory results can be obtained by this method.

TABLE III.

HALF-CYSTINE PLUS CYSTEINE CONTENT OF PROTEINS

These values were determined by extrapolation to zero protein conc. by plots similar to those given in Fig. 4. The values were calculated by the method of least squares.

Protein	Source	$E^{1\%}_{1\ cm}$	Assumed Molecular Weight	Half-cystine + cysteine			
				Experimental	Literature		
Bovine serum albumin (cryst.)	Armour Lot #128-176	6.60 (114)	69,000	34.9±0.5	35	(52)	
Trypsin (cryst.)	Worthington Biochemical Corp. Lot. TRSF-688	14.4 (115)	24,000	12.0±0.2	12	(97)	
Ribonuclease (cryst.)	Worthington Biochemical Corp. Lot. No. 558	6.9 (116)	13,683	8.0±0.2	8	(117)	
Insulin (bovine)	Squibb, Zn-free	10.0 (118)	5,700	5.9±0.1	6	(119)	
Ovalbumin (cryst.)	Nutritional Biochemical Corp. 2xcryst., salt-free	7.35 (120)	45,000	7.0±0.1	6.5-7	(121)	
Lysozyme (cryst.)	Armour Lot No. 003L1	27.3 (122)	14,000	10.4±0.4	8 10	(123); (86,124)	

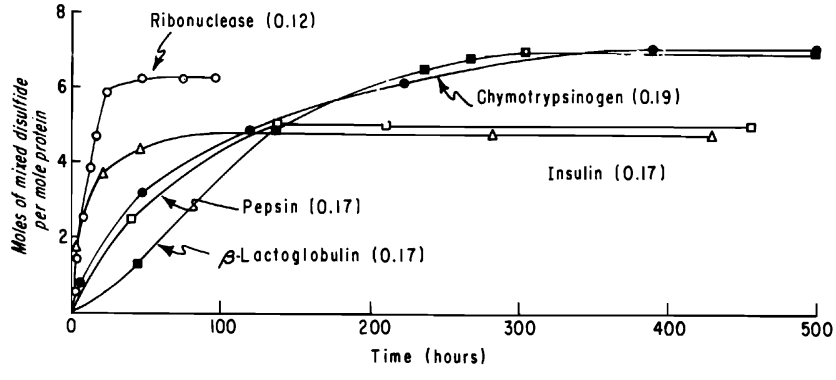

Fig. 2. Reaction of several proteins with di-DNP-cystine in 9.6N HCl at 39°. The concentration of the protein is given parenthetically in μmole/ml.

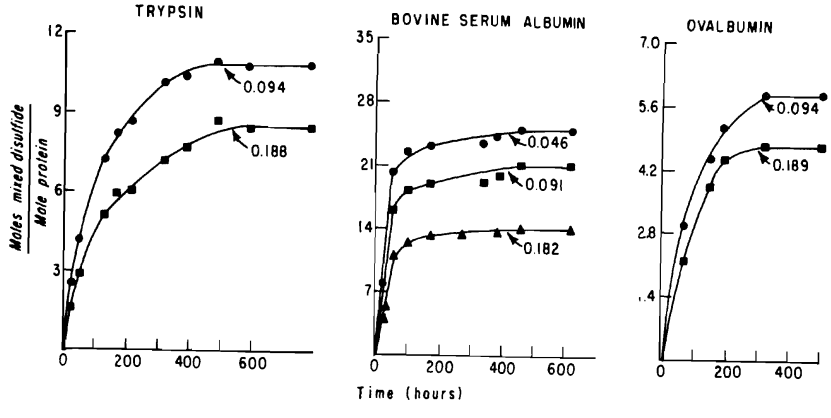

Fig. 3. Effect of protein concentration on rate and equilibrium of reaction with di-DNP-cystine in 9.6N HCl at 39°. Protein concentrations are given in μmole/ml.

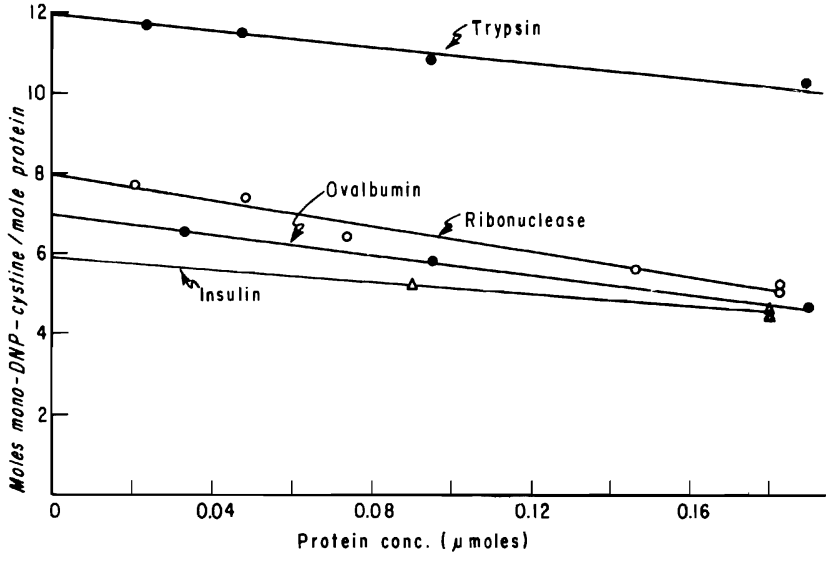

Fig. 4. Equilibrium values for formation of mono-DNP-cystine as a function of protein concentration.

Specificity of the Reaction: No reaction with any amino acid other than cystine or cysteine could be observed under the conditions described above. Methionine did not react at all, regardless of concentration used. Proteins known to be free of cystine and cysteine, e.g., whale myoglobin and protamine sulfate (salmine), did not give any interchange (Table IV). Perhaps the strongest evidence supporting the view that the interchange reaction is completely specific for cystine (and cysteine), under the conditions used in these experiments, is the fact that the results obtained with proteins of known sulfur content are in complete accord with those reported in the literature.

DISCUSSION

When this investigation was initiated, it was hoped that it would be possible to distinguish between disulfide and sulfhydryl content, or at least between proteins containing only disulfide bonds, and those containing both disulfide bonds and sulfhydryl groups, on the basis of the rates of the interchange reaction. It is evident from inspection of the figures that this did not prove to be possible. Thus, for example, trypsin which contains six disulfide bonds and no sulfhydryl groups reacts with di-DNP-cystine in the interchange reaction far more slowly than ovalbumin which has only one disulfide bond and four or five sulfhydryl groups.

Under the conditions used in this study, the proteins may be considered to undergo essentially complete denaturation immediately on coming in contact with the 9.6N HCl. Indeed, the proteins also undergo extensive hydrolytic cleavage within a short time. It is clear, therefore, that differences in the reactivity of the disulfides in different proteins cannot be ascribed to effects based on the presence of secondary structure.

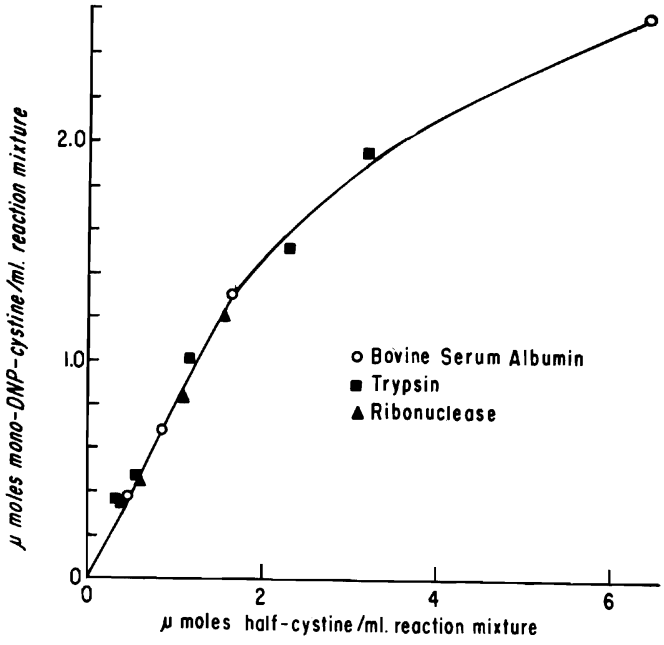

Fig. 5. Equilibrium values for formation of mono-DNP-cystine as a function of half-cystine (plus cysteine) concentration of the protein in the reaction mixture.

TABLE IV

HALF-CYSTINE PLUS CYSTEINE CONTENT OF PROTEINS

These values were determined by duplicate estimations of mono-DNP-cystine formed at a single protein conc. The protein content of half-cystine plus cysteine was then estimated by interpolation on the standard curve of Fig. 5.

Protein	Source	$E^{1\%}_{1\ cm}$	Assumed Molecular Weight	Half-cystine + cysteine	
				Experimental	Literature
Pepsin (porcine)	3x crystallized	14.3 (125)	35,500	6.0±0.3	6 (97,2)
β-Lactoglobulin	3x crystallized	9.5 (18)	35,500	9.8±0.2	10 (103)
Chymotrypsinogen (cryst.)	Armour R337254	20.0 (70)	25,100	10.1±0.3	10 (70)
Myoglobin, whale (recryst.)	Gift of Dr. J. C. Kendrew	*	17,000	0.1	0 (126)
Human γ-globulin (II-1,2)	Squibb	*	160,000	35.1±0.8	34.6 (127)+
Protamine sulfate	Squibb	*	8,000	0.0	0 (128)

* Protein content was determined by dry weight.

\+ This value is based on a molecular weight of 160,000 and a half-cystine plus cysteine content of 2.6% calculated from the total sulfur minus the methionine sulfur of this preparation.

Rather, the explanation probably resides in the influence of neighboring polar groups in the peptides containing the disulfide. Such polar groups may either labilize the S-S bond or render it less active. Part of the effect exerted by the neighboring groups may, of course, be due to repulsion of the positively charged attacking sulfenium ion (RS^+) and also of protons when positively charged ϵ -ammonium groups of lysine or guanidinium groups of arginine are in close proximity to the disulfide bond.

The importance of electrostatic effects has been shown by Moore and Porter (129), who studied the exchange reaction of arenesulfenyl chlorides (e.g., 2:4-dinitrophenylsulfenyl chloride) with organic disulfides. These investigators demonstrated that the rate of exchange at room temperature was dependent on the polarity of the solvent, exchange being considerably faster in acetic acid than in less polar solvents, such as ether, carbon tetrachloride and xylene.

The differences in the rate of interchange found with the various proteins under conditions where all secondary structure and much of the primary have been destroyed, illustrate the great importance of the neighboring amino acids in the peptide chain in determining the reactivity of the disulfide bond.

In view of the mechanism proposed by Benesch and Benesch (110) for the interchange reaction, the interchange obtained with thiols may appear to be unexpected at first glance. It should be noted, however, that no attempt was made in the present studies to exclude either oxygen or trace metals from the reaction mixture and the reaction observed may be due to slow oxidation of thiol groups to disulfides, which can then participate in the interchange reaction. The above interpretation is also consistent with the finding that for proteins such as β-lactoglobulin, ovalbumin, etc., which

contain both disulfide and thiol groups, the values obtained from the interchange reaction represent the total of the disulfide and sulfhydryl content.

It should be mentioned that if sulfenic, thiol ester or thiazoline groups exist in proteins, which are capable of either reacting directly or giving rise to sulfhydryl and hence disulfide under the conditions of the interchange reaction, such groups would be included in the mixed disulfide value as determined by this method.

The agreement of the values obtained in this study with those reported in the literature is excellent. In the case of γ-globulin the value of 35.1 ± 0.8 obtained for the cystine plus cysteine content of this protein agrees well with the value of 34.6 calculated from the total sulfur content minus the methionine sulfur present in this protein. The data shown in Fig. 4 shows that for proteins with 3-6 disulfide bonds, e.g., insulin, ovalbumin), two or three points suffice to obtain a reliable extrapolated value. Therefore, it is possible to obtain such a value with as little as 0.6-1 μmole of protein.

Although earlier work had indicated that egg white lysozyme contains 10 half-cystine residues (86,124), Jolles *et al.*(123) have indicated recently that this protein may contain only 8 such residues. It is noteworthy that the results indicate 10 half-cystine residues in lysozyme in accord with several earlier studies.

Since the equilibrium values for mono-DNP-cystine concentrations obtained with the different proteins studied fall fairly well on the curve shown in Fig. 5 it would appear that a similar equilibrium is reached in all cases. This is not surprising in view of the fact that by the time equilibrium is reached the hydrolysis of each

of the proteins has progressed to the point where essentially only dipeptides and free amino acids are present.

It is not expected that the present method will displace others currently available for the determination of cystine plus cysteine in proteins. Rather, the method may provide a useful additional method for this determination. One of the methods most widely used at present for total cysteine plus cystine is that of Schram, Moore and Bigwood (71). Their procedure and its various modifications involve oxidation with performic acid, hydrolysis under standard conditions, and determination of cysteic acid by a chromatographic separation. The major uncertainty in this oxidative method is the size of the correction factor which must be applied to provide for the hydrolytic loss of cysteic acid. It is evident from the exchange results that no correction factors are needed nor are any chromatographic separations required. The main drawback to the method would appear to be the patience required to wait the several weeks which may be necessary for the attainment of equilibrium.

Summary of Results

The disulfide interchange reaction of di-DNP-cystine in concentrated acid solution with cystine, simple thiols and a number of proteins has been investigated. The effect of acid concentration, temperature, and protein concentration on the kinetics and equilibrium of the exchange reaction has been studied. The reaction appears to be specific for the disulfide and thiol groups of proteins. No reaction with methionine or with proteins lacking sulfur-containing amino acids could be demonstrated. The various proteins studied varied widely with respect to the rate of exchange with di-DNP-cystine. The equilibrium nature of the reaction was utilized in

the development of a method for the precise estimation of the half-cystine plus cysteine content of proteins. The results obtained are in good agreement with those in the literature for the half-cystine plus cysteine content of 12 different proteins.

C. APPLICATION OF THE DISULFIDE INTERCHANGE REACTION TO PAPAIN

The behavior of papain in the disulfide interchange reaction was similar to that of the other proteins discussed in the preceding section. The rate of interchange was found to be intermediate between that found with ribonuclease and that found with trypsin. Figure 6 shows representative rates of interchange at different papain concentrations.

The equilibrium concentration of the mixed disulfide was determined in the protein concentration range 0.04-0.20 μmoles per ml of reaction mixture. The results are shown in Figure 7. From this figure, it may be seen that extrapolation to zero protein concentration yields a value of eight groups capable of participating in the exchange reaction per mole of papain.

This result excludes the possibility that the two "unknown" sulfur atoms in papain may be present as bound inorganic sulfate or sulfate esters which would break down to yield sulfate on acid hydrolysis.

Further, since the behavior of papain in the exchange reaction is very similar to that of other proteins of known composition as far as the sulfur-containing amino acids are concerned, the results are consistent with the view that the eight sulfur atoms in papain are present in for form of disulfide and sulfhydryl groups. This, of course, does not necessarily mean that these eight groups represent eight half-cystine residues.

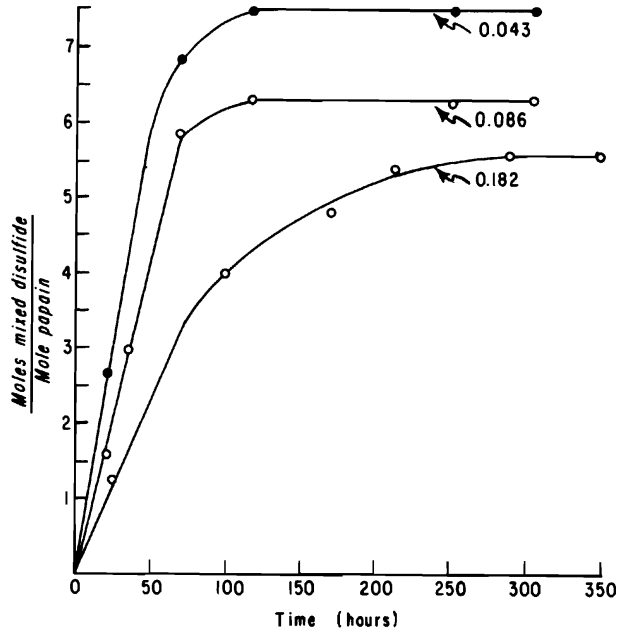

Fig. 6. Effect of papain concentration on rate and equilibrium of reaction with di-DNP-cystine in 9.6N HCl at 39°. Protein concentrations are given in μmole/ml.

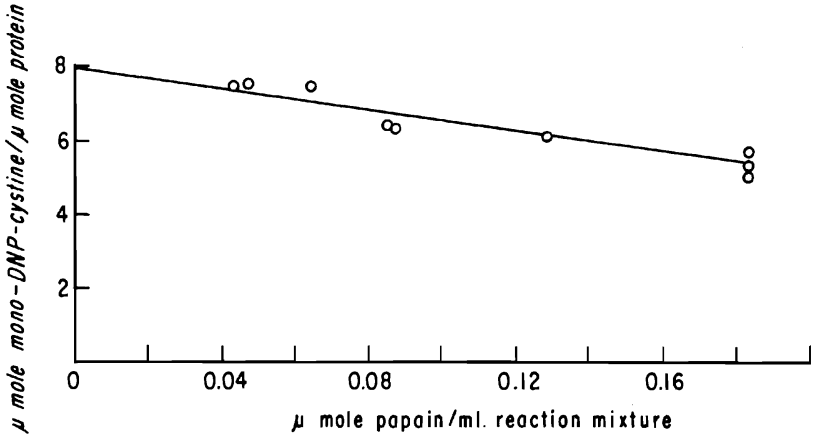

Fig. 7. Equilibrium values for the formation of mono-DNP-cystine as a function of papain concentration.

D. DISULFIDE CONTENT OF PAPAIN

The study of papain with the sulfhydryl reagents indicated clearly the presence of less than 0.5 moles -SH/mole of papain. The application of the exchange reaction demonstrated, however, that papain possessed eight exchangeable groups and that these were most probably in the form of S-S and -SH. The data presented in this section represents an investigation of the disulfide content of papain. The presence of disulfide bonds is clearly indicated by the data presented in sections A-C.

a. Materials.

All of the materials used were described in Section A-a.

b. Methods.

Determination of Sulfhydryl and Disulfide Groups by Amperometric Titration

The apparatus described by Benesch, Lardy and Benesch (43) was used. The length of the bridge carrying the electrolyte solution was reduced to 30 cm. A platinum electrode rotated by a synchronous motor at 600 r.p.m. was used throughout. The reference electrode was the Hg-HgO - saturated $Ba(OH)_2$ electrode of Samuelson and Brown (133) with a potential of -0.10 volt *vs.* standard calomel electrode. The electrode was shielded from light as suggested by Benesch *et al.* (43). A schematic representation of the apparatus (Figure 9), and a key to the parts are presented at the end of this section. 1×10^{-3}M silver nitrate was used for all of the titrations. A reduced glutathione standard was routinely included in each set of determinations. Titrations of reduced glutathione gave recoveries in the range 98±3 per cent. Several different sets of conditions were used for the sulfhydryl and disulfide determinations.

(1) Titrations in Tris buffer, pH 7.4.

These were carried out by the method of Benesch *et al.* (43). The reaction mixture contained 4.0 ml 1.0 M tris (hydroxymethyl) aminomethane,

3.4 ml 1.0 M HNO_3, 0.2 ml 1.0 M KCl, the denaturing agent in the concentration specified, and 0.1-0.5 μmoles protein. The final volume was 30.0 ml and the pH 7.4. The above mixture was used for the titration of -SH groups. For the determination of disulfide groups, 0.1 ml freshly saturated Na_2SO_3 was added. Titration was commenced 20 seconds after the addition of sulfite. The values for disulfide were obtained by subtracting the -SH values from the values obtained by sulfitolysis.

(2) "Excess Reagent" Method.

The reaction mixture exactly as described above was used in the "excess reagent" method (43) with the addition of a predetermined excess of silver nitrate solution. 8M urea was used as a denaturing agent. Experiments were carried out both in the absence and presence of sulfite. After the solution was kept in the dark at 39° for varying periods of time, it was cooled to room temperature and titrated as usual.

(3) Titrations in 8M urea at pH 8.4.

These titrations were carried out by the method of Carter (97). The reaction mixture contained in addition 0.1 ml 1:10 diluted saturated Na_2CO_3, while for the determination of S-S content, 0.1 ml freshly saturated Na_2SO_3 was added. The pH of the reaction mixture was in the range 8.3-8.5. Titration was commenced 20 seconds after the addition of sulfite and carried out at 37° as recommended by Carter (97). Pepsin and trypsin were used as controls in the titrations carried out by this method. The values obtained for the disulfide content of these proteins were 3.0±0.1 and 5.8±0.1 respectively, in good agreement with those reported by Carter (97).

(4) Titration of Acid-Denatured Papain.

Three mls of papain solution containing 0.1-0.5 μmoles papain were adjusted to pH 1.7 with 1M HCl and allowed to stand at room temperature

for varying periods of time. The acid solution was then mixed with a urea-Tris buffer, pH 7.4, to give a final volume of 30.0 ml and final urea concentration of 8M, 0.1 ml freshly saturated Na_2SO_3 was then added and titration commenced immediately.

c. Results.

The conditions used for the amperometric titrations and the results obtained are given in Table V.

(i) Titrations in Tris buffer, pH 7.4.

In absence of denaturing agents, papain gives a titer of approximately three disulfide groups per mole of protein. This indicates that three of the disulfide bonds in papain behave towards sulfite in a manner analogous to that of simple model disulfides (55,95). In the presence of sodium lauryl sulfate, or 8M urea, a somewhat lower titer of 2.5 S-S bonds per mole was obtained.

(ii) Titrations in Tris Buffer, pH 7.4, and 8M urea, by the "Excess Reagent" Method.

When the titrations were carried out by the excess reagent method (43) in the presence of 8M urea, 2.9-3.0 moles of disulfide per mole of papain were titrated. Since the disulfide titer obtained by this method was independent of the silver ion concentration within the range 2.5 to 20.0 moles of silver nitrate per mole of papain, it would appear unlikely that any anomalous binding of silver ions was taking place under the conditions used.

(iii) Titrations in 8M urea, pH 8.4.

By following precisely the procedure of Carter (97), and carrying out the sulfitolysis and titration at 37°, a titer of approximately 2.4 moles of S-S per mole of papain was obtained. The variation between duplicate

titrations was of the order of 10 per cent. On the whole, the results obtained by Carter's method are in accord with those obtained at pH 7.4.

(iv) Titration of Acid-Denatured Papain.

Urea is known to be a poor denaturing agent for papain whereas acid is a very good one (4). A combination of acid-denaturation followed by denaturation in 8M urea was, therefore, employed to ensure extensive disorganization of the papain molecule. After a 30 minute period at pH 1.7, the solutions were mixed with a Tris-urea mixture to give a final urea concentration of 8M and pH 7.4. As a result of this treatment, four moles of disulfide per mole of papain could be titrated on addition of 0.1 ml freshly saturated sulfite (Figure 8).

d. Discussion.

Since only three disulfide bonds react with sulfite even when the protein is treated with urea or sodium lauryl sulfate at near-neutral pH, it appears that the fourth bond must be located in an unfavorable environment for sulfite cleavage (55,134). It may be located in a portion of the papain molecule which is tightly coiled and disorganized to only a small extent by urea or sodium lauryl sulfate. Presumably, the use of acid which is known to denature papain irreversibly, is required to unfold this portion of the molecule and change the reactivity of the disulfide bond within it towards sulfite. The data obtained from the extensive degradation of mercuripapain by leucine aminopeptidase does indicate that a large portion of the molecule appears to be relatively loosely coiled and a portion quite tightly coiled (10). Cecil and Loening (55) have demonstrated that in insulin two of the three disulfide bonds react with sulfite in a manner analogous to that of simple disulfide compounds (95). The cleavage of the third bond requires prior denaturation of insulin by urea (55). Cecil has

shown that the reactivity of model disulfides depends on the overall charge of their environments (134). Those with a positive environment are cleaved very slowly. He has suggested (134) that this could provide an explanation of the lack of reactivity of the third disulfide bond in native insulin. A similar explanation may apply to the difficulty experienced in cleaving the fourth disulfide bond in papain.

TABLE V

AMPEROMETRIC TITRATIONS ON PAPAIN

		Titration Temp.	No. of Determinations	Moles S-S / Mole papain	Moles -SH / Mole papain
Titrations in Tris buffer, pH 7.4					
No denaturing agent		25°	5	2.8 ± 0.3	0.1 - 0.25
0.6% sodium lauryl sulfate		25°	3	2.5 ± 0.2	0.1 - 0.2
8M urea		25°	5	2.5 ± 0.2	0.1 - 0.2
Excess Reagent Method					
Denaturing agent: 8M urea in Tris buffer, pH 7.5					
$AgNO_3$ added initially per mole papain, moles	Time of incubation at 39°, min.				
2.5	25	25°	1	3.0	0.2
7.5	40	25°	1	2.9	0.2
20.0	20	25°	1	2.9	0.2
Titrations in 8M urea, pH 8.4					
		37°	3	2.4 ± 0.2	0.1 - 0.2
Titration of Acid-Denatured Papain					
	Time of exposure to acid, min.				
	5	25°	2	3.6 ± 0.1	0
	15	25°	2	3.85 ± 0.1	0
	30	25°	5	4.0 ± 0.1	0

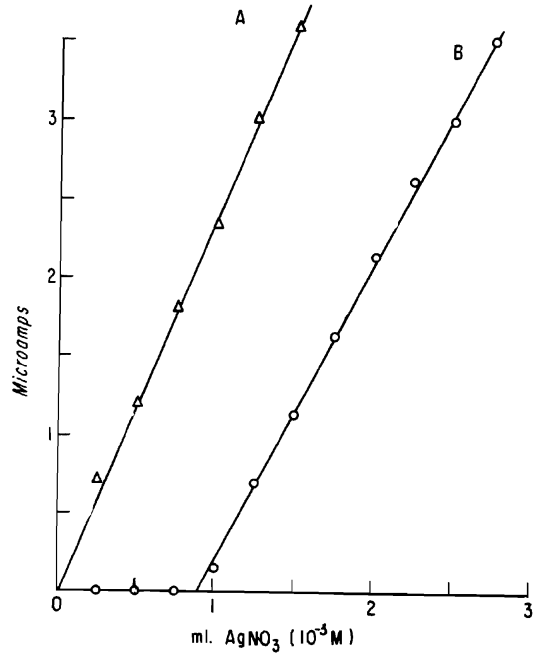

Fig. 8. Amperometric Titration Curves of Acid-Denatured Papain. Curve A: In 8M urea and Tris buffer, pH 7.4. Curve B: In 8M urea and Tris buffer, pH 7.4, after the addition of 0.1 ml saturated sodium sulfite. The titration mixture contained 0.23 μmoles of papain. For procedure used, see "Results", section D-c (iv).

AMPEROMETRIC TITRATION ASSEMBLY

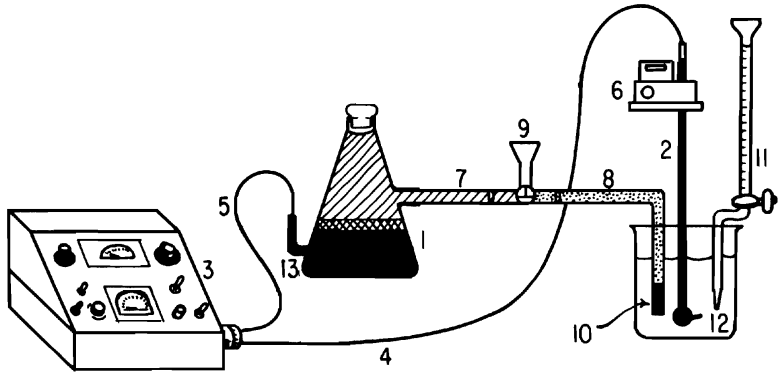

Figure 9.

KEY TO AMPEROMETRIC TITRATION ASSEMBLY. FIGURE 9.

1. Reference Electrode: 250 ml capacity thick-walled conical flask with side-arm A short platinum wire fused through the flask near the base serves to make the connection with the negative lead from the microammeter. The three layers in the flask are (a) layer of mercury at the bottom, which serves as the electrode and into which the platinum wire mentioned above dips, (b) a slurry of solid HgO and $Ba(OH)_2$, and (c) a saturated supernatant solution which fills up the flask. This electrode was protected from light with several layers of aluminum foil.

2. Rotating Platinum Microelectrode (S-30420). This electrode is in the form of a platinum wire sealed in the side of a 10 mm bulb on the end of a piece of 6 mm soft glass tubing. This electrode is mounted in the chuck of the Sargent Synchronous Rotator (see 6 below). Electrical connection to the electrode is made by filling the bulb portion and half the tubing section of the electrode with mercury and inserting a lead wire into the mercury. The other end of the wire is connected to the Electrometric Titration Instrument (see 3 below). For solutions containing precipitates, the hook-type electrode (S-30421) is used.

3. Electrometric Titration Instrument (Sargent, "Ampot", S-29710) equipped with a dial type microammeter provided with appropriate shunts to provide current sensitivities of 0.5, 1.0, 2.5, and 5.0 microamperes per scale division.

4. Shielded lead wire with nickel terminal (NEGATIVE lead).

5. Shielded lead wire with nickel terminal (POSITIVE lead).

6. Sargent Synchronous Rotator (S-76485) designed for operation from 115 volt 60 cycle A.C. circuits. Drives the chuck at a constant speed of 600 r.p.m.

7. 30 cm Tygon tubing bridge containing electrode solution.

8. 30 cm Tygon tubing bridge containing saturated KCl. Internal diameter of the Tygon tubing in both 7 and 8 is 6 mm. (internal diameter).

9. 15 ml capacity funnel equipped with a three-way stopcock.

10. Short piece of glass tubing equipped with a Whatman No. 1 filter paper plug.

11. Microburette (S-10921) with elongated offset burette-tip. Capacity is 10 ml with 0.05 ml gradations.

12. 50 ml beaker containing titration mixture.

13. Glass tube fused onto reference electrode over point of emergence of platinum tube and half-filled with mercury. The negative lead wire is inserted into this tube.

Catalog numbers refer to E. H. Sargent and Co., Catalog No. 100, 1953 Editio

E. CYSTEIC ACID CONTENT OF OXIDIZED PAPAIN

The results obtained by the exchange reaction and the disulfide determination after sulfitolysis indicate strongly the presence of eight half-cystine residues in papain. However, the estimation of cysteic acid in hydrolysates of performic acid oxidized papain indicated the presence of only six residues of half-cystine per mole of the enzyme (68,69). Two explanations could be put forward to account for this discrepancy. Firstly, performic acid oxidation of proteins frequently leads to partial oxidation products between the half-cystine and the cysteic acid oxidation levels. Such intermediates dismute to give half-cystine and cysteic acid on acid hydrolysis (72). Since cystine was not estimated in the earlier work, no evaluation of this possibility was available. Secondly, it was possible that one of the disulfide bonds in papain was sterically poorly available to oxidation. Such an explanation is not inconsistent with the information presented in the preceding sections.

To examine these possibilities, the cysteic acid content of papain which had been denatured with acid and treated with sulfite was determined after performic acid oxidation.

METHODS AND RESULTS

Preparation of Acid-treated Sulfitolized Papain

30.0 mg lyophilized papain were dissolved in 5 ml water, pH 1.7 (HCl), and allowed to stand 30 min at 25°. A solution of urea in Tris buffer pH 7.6 was added to a final volume of 30 ml and urea concentration of 8.0 M. 1.0 ml of freshly saturated Na_2SO_3 was added and the solution stirred rapidly for 5 min. The solution was transferred to a dialysis bag and dialyzed against frequent changes of deionized water for 24 hours at 0°. The papain precipitated out of solution on removal of the urea. The protein

precipitate was centrifuged down and washed several times in the centrifuge with deionized water. The washed precipitate was then lyophilized. Recovery of the starting material was 88 per cent at this point.

Performic Acid Oxidation: To the lyophilized precipitate added 4 ml 99 per cent formic acid (Coleman-Bell) and 1 ml absolute methanol in a salt-ice bath (65). A twenty-fold excess of precooled performic acid (calculated on the basis of 8 sulfhydryl groups per mole of papain) was then added and the mixture allowed to react at -10° for 3 hours. The mixture was then diluted with ten volumes of distilled water and lyophilized. The lyophilization was repeated twice more.

Acid Hydrolysis: The lyophilized material was divided into three portions and hydrolyzed in 6N HCl at 105° for 20, 50 and 66 hours. Suitable aliquots of the hydrolysates were then analyzed on the Amino Acid Analyzer. The results of the amino acid analyses are presented in Table VI.

Determination of the Cysteic Acid Content of Oxidized Papain by the Method of Moore.

In a recent personal communication to Dr. Emil L. Smith, Dr. S. Moore has outlined a new modified procedure for the determination of cystine plus cysteine as cysteic acid. The instructions given were as follows:

"Weight 5-10 mg of protein into an 18 x 150 mm Pyrex test tube (preferably heavy-walled, Corning No. 9860). Add 2 ml of cold pre-formed performic acid (1 ml of 30 per cent H_2O_2 added to 9 ml of 88 per cent formic acid, allowed to stand 1 hour at room temperature, and then cooled to 0°.)

"Let stand for 4 hours at 0° for soluble proteins, overnight for insoluble ones.

"Then add slowly, with swirling in the 0° bath, 2.5 ml of freshly

prepared about 10 per cent sodium bisulfite solution (1 gm + 10 ml H_2O).

"Concentrate the solution to dryness on the Craig rotary evaporator. To the tube add 3 ml of 6 N HCl. Take the solution to dryness again, to remove SO_2. Take up the residue in 2-3 ml of 6 N HCl. Freeze, evacuate, seal and hydrolyze as usual.

"Remove HCl on the rotary evaporator. Add 5.0 ml of pH 2 buffer and place a 2 ml aliquot on the 150 cm column of the amino acid analyzer.

"We usually calculate the cysteic acid as a molar ratio relative to the amounts of other amino acids the content of which is already known from prior analyses of the unoxidized protein. This means that we do not need to have moisture and ash on the sample for oxidation nor make the final solution exactly to volume. All operations are carried out in the one tube; no transfers are required.

"The yield of cysteic acid is 90 per cent, and the result is corrected for this yield. The use of bisulfite in this way serves to destroy all of the oxidizing agent before the solution is warmed up or concentrated, and this eliminates the possibility of any loss arising from over-oxidation in the concentration step.

"With ribonuclease A the procedure gives 8.04 residues of half-cystine per molecule.

"The yield of methionine sulfone is 100 per cent.

"The original procedure of Schram *et al.* gave good results when the directions were followed carefully, but there can be low results if the performic acid solution is concentrated all the way to dryness, as pointed out in the paper. The modified procedure removes this hazard and provides directions that give reproducible results without any step that requires special care or experience."

The above method was applied to the determination of the half-cystine content of papain. The only departure from the instructions as stated above was that following the addition of sodium bisulfite solution and then 6N HCl the mixtures were dried in a vacuum desiccator over NaOH. Two papain preparations were oxidized and hydrolyzed for 20 and 70 hours, respectively. The cysteic acid was calculated as a molar ratio relative to the amounts of other amino acids, the content of which was already known from prior analyses (See Fig. 10 for representative amino acid analysis). The tyrosine value was not used in the mean residue value computation. A 25-30 per cent loss of this residue took place under the conditions used for the oxidation. The values obtained from the analyses of 20 and 70 hour hydrolysates of the two preparations are listed in Table VII. These results yield a mean value of 7.7 $\pm$ 0.1 residues of cysteic acid per mole of papain. Clearly, this represents a higher yield than that obtained by Moore with ribonuclease A. The cysteic acid yield in the case of papain appears to be 96 per cent. As may be seen from Fig. 10, no cystine could be detected in the 70 hour hydrolysate.

DISCUSSION

The results of oxidation of sulfite-treated acid-denatured papain by the method of Hirs (65) indicates the presence of at least seven half-cystine residues in the papain molecule. The presence of cystine in the 70 hour hydrolysate is indicative of incomplete oxidation.

The results obtained with the modified method of Moore clearly show the presence of 7.7 $\pm$ 0.1 residues of cysteic acid per mole of papain without application of any correction factors. This value closely approaches the theoretical value of 8 (assuming that the 8 sulfur atoms in papain are exclusively contained in half-cystine residues). Presumably, the low results

obtained in previous studies (68,69) were due to overoxidation of the cysteic acid to sulfate.

CONCLUSIONS

Amino acid analyses of acid-denatured sulfite-treated performic acid oxidized papain after acid hydrolysis under standard conditions show the presence of 6.1 moles of cysteic acid and 0.8 moles of half-cystine per mole of enzyme. No correction for destruction has been applied to these values.

Amino acid analysis of acid hydrolysates of papain oxidized with performic acid by the modified procedure of Moore yields a value of 7.7 $\pm$ 0.1 moles of cysteic acid per mole of papain.

It appears, therefore, that the sulfur content of papain can be accounted for in terms of the half-cystine content of the protein.

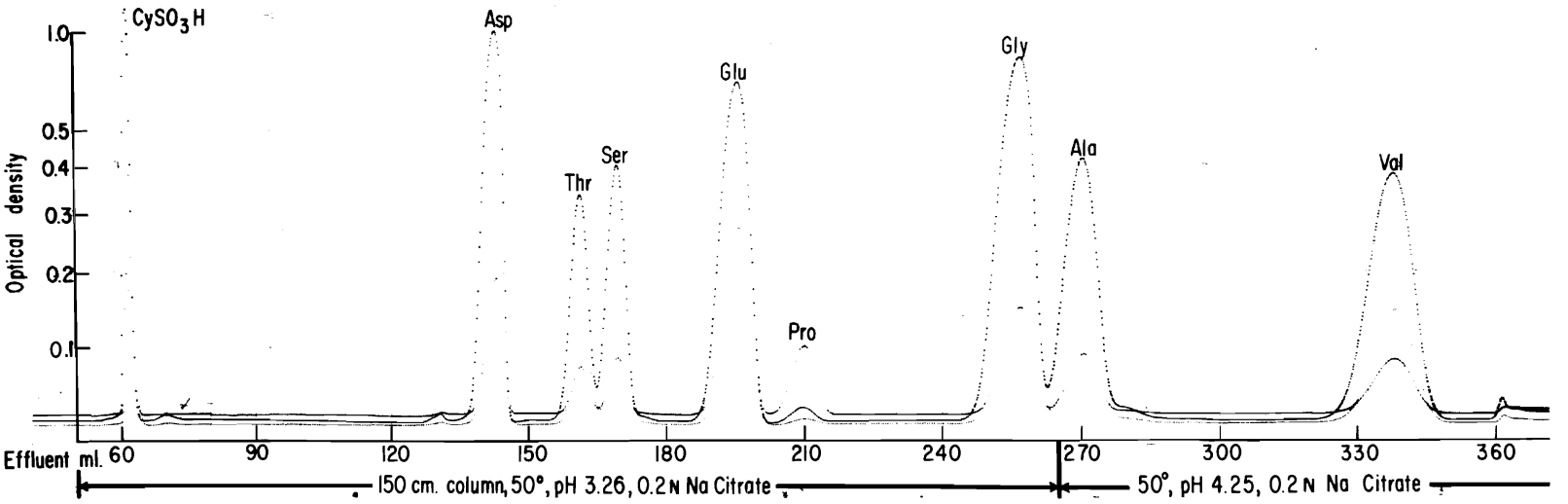

Fig. 10. Amino Acid Analysis of 70-hour hydrolysate of oxidized papain.

Oxidation was performed by the modified method of Moore.

TABLE VI

Cysteic Acid Determinations on Acid-Denatured Sulfite-Treated Papain Oxidized by the Method of Hirs (65).

Time of hydrolysis* (hours)	Moles cysteic acid / Mole papain	Moles cystine/2 / Mole papain
20	6.06	0.80
50	6.14	-
66	6.02	0.34

TABLE VII

Cysteic Acid Determinations on Papain Oxidized by the Modified Method of Moore.

	Time of hydrolysis* (hours)	Moles cysteic acid** / Mole papain
Prep. No. 1	20	7.5
	70	7.6
Prep. No. 2	20	7.8
	70	7.8

* 6N HCl, at 110°, in evacuated tubes.

** Cystine was absent from these hydrolysates.

F. EFFECT OF DIISOPROPYL FLUOROPHOSPHATE ON PAPAIN

Many esterases have the property of reacting with diisopropyl fluorophosphate (DFP) to form an enzymically inactive compound. When a number of DFP-inhibited enzymes were hydrolyzed with proteolytic enzymes, the diisopropylphosphoryl residue was found to be attached to the hydroxyl group of a serine residue (135-139). A number of reasons have been put forward to support the contention that DFP reacts specifically with the active site. The enzymes concerned are inhibited at low concentrations of DFP. One mole of DFP per mole of enzyme is sufficient to give complete inhibition. Further, when inhibition is only partial, its degree is always linearly related to the amount of diisopropylphosphoryl-bound. Lastly, it was noted that substrates prevent inhibition by DFP indicating competition for a common active site.

Since papain is an esterase, its behavior towards DFP is of considerable interest. The effect of DFP on the proteolytic activity of papain was first examined by Jansen, Nutting and Balls (140). At a DFP concentration of 10^{-3}M these investigators found less than 10 per cent inhibition of papain. Heinicke and Mori (141), on the other hand, have recently claimed that incubation of both papain and bromelain with 10^{-3}M DFP for one hour at 25° resulted in an extensive inhibition of these enzymes. It may be noted that the proteins were incubated with the DFP in the absence of activators in this study. Murachi and Neurath (142) were unable to detect any inhibition of bromelain under the conditions specified by Heinicke and Mori (141). More recently, Masuda (143) has claimed a 100 per cent inhibition of cyanide-activated papain with 10^{-3}M DFP. He could obtain no inhibition of the untreated enzyme or of the enzyme activated with cysteine, thioglycolate, or hydrogen sulfide.

Much evidence is available that papain is a sulfhydryl enzyme (4,15,22) and as such would not be expected to be inhibited by DFP. The alleged inhibition with DFP was, therefore, investigated with the synthetic substrates benzoylargininamide, benzoylarginine ethyl ester and p-toluenesulfonylarginine methyl ester.

Materials.

Papain was prepared by the method of Kimmel and Smith (105). It had a C_1 of 1.6 towards benzoylarginine amide at pH 5.1 and 1.0 towards p-toluene sulfonylarginine methyl ester at pH 5.3 with the Grassman-Heyde method of assay (106) and cysteine as activator. Protein concentrations were determined spectrophotometrically.

Benzoylargininamide (BAA) and benzoylarginine ethyl ester (BAEE) were prepared by the method of Kimmel and Smith (105). p-Toluenesulfonylarginine methyl ester was obtained from the H. M. Chemical Co., Ltd., (C.P. grade) and used without further purification.

Di-isopropylfluorophosphate (DFP) was obtained from Merck in sealed 1 ml ampules. A fresh 0.1M DFP solution was prepared in isopropanol and used within 4 days. When assayed against trypsin, it was found that 1.3 moles of DFP per mole of trypsin produced a 98 per cent inhibition of this enzyme.

Methods.

The Grassman-Heyde method (106) was used for the assay of papain with BAA as substrate. The pH-stat was used for the assays involving the ester substrates. The assays were carried out in 0.05M phosphate buffer, 0.1 or 0.2M cyanide at pH 7.4 and 39°. The details of DFP concentration and cyanide concentration as well as details of length of incubation are listed in Table VIII.

RESULTS AND DISCUSSION

With cyanide as activating agent under the conditions specified by Masuda (143), no inhibition by DFP could be demonstrated over the concentration range 10^{-6} to 10^{-3}M. The results are presented in detail in Table VIII.

In order to be able to assay papain in presence of 10^{-2}M DFP it was necessary to add the inhibitor in a volume of isopropanol such that the final concentration of the alcohol was 10 per cent. Under these conditions 80 per cent inhibition of papain was observed. However, the degree of inhibition was the same whether the isopropanol contained DFP or not.

Isopropanol inhibition of papain was reported by Stockell and Smith (19). The inhibition was shown to be caused by the lowering of the dielectric constant of the medium (19). The inhibition observed in the present study at pH 7.4 is much greater than that reported by Stockell and Smith (19) at pH 5.2. It is possible that at the higher pH value, the inhibition is only partly due to the decreased dielectric constant of the medium and that some transesterification between the p-toluenesulfonylarginine methyl ester and the isopropanol takes place under these conditions.

CONCLUSIONS

1) Cyanide-activated papain is not inhibited by DFP in the concentration range 10^{-6} - 10^{-3}M. No inhibition was observed even after prolonged incubation of the activated papain with the inhibitor.

2) Isopropanol inhibits papain under the conditions used for cyanide activation. It is possible that this inhibition is in part due to transesterification between the isopropanol and the ester substrate.

TABLE VIII

EFFECT OF DFP ON CYANIDE-ACTIVATED PAPAIN

Substrate	Incubation Mixture			Incubation Time	Assay Mixture			
	DFP(M)	CN^-(M)	IP(%)*	(minutes)	DFP(M)	CN-(M)	IP(%)*	C_1
BAA	7.5×10^{-5}	0.2	1.0	5	7.5×10^{-6}	0.2	0.1	0.59
	-	0.2	-	5	-	0.2	-	0.65
BAEE	7.5×10^{-5}	0.1	1.0	5	7.5×10^{-6}	0.1	0.1	0.75
	7.5×10^{-4}	0.1	10.0	60	7.5×10^{-5}	0.1	1.0	0.75
	-	0.1	-	60	-	0.1	-	0.88
	1.0×10^{-3}	0.2	10.0	20	1.0×10^{-4}	0.2	1.0	0.88
	1.0×10^{-3}	0.2	10.0	50	1.0×10^{-4}	0.2	1.0	0.90
	1.0×10^{-3}	0.2	10.0	150	1.0×10^{-4}	0.2	1.0	0.91
	-	0.2	-	5	-	0.2	-	1.05
	-	0.2	-	150	-	0.2	-	0.90
TAME	-	0.2	-	5	-	0.2	-	1.05
	1.0×10^{-2}	0.2	10.0	5	1.0×10^{-2}	0.2	10.0	0.10
	-	0.2	10.0	5	-	0.2	10.0	0.09
	1.0×10^{-2}	0.2	10.0	25	1.0×10^{-3}	0.2	1.0	0.54
	-	0.2	10.0	25	-	0.2	1.0	0.50

* IP = isopropanol.

Papain concentrations were in the range 0.10-0.40 mg per ml assay mixture.

GENERAL DISCUSSION

The determination of the sulfhydryl content of unactivated papain with p-mercuribenzoate, N-ethylmaleimide, as well as silver ion, consistently indicates the presence of less than 0.5 mole of sulfhydryl per mole of enzyme. The titer cannot be increased by any of the several methods of denaturation which were examined in the course of this work.

The presence of sterically masked sulfhydryl groups in papain has been definitely ruled out by these studies. It is clear that the binding, under certain special circumstances, of four to six moles of PCMB per mole papain reported by Finkle and Smith (15) cannot represent reaction with sulfhydryl groups as such. Finkle and Smith (15) determined the amount of PCMB reacting with papain by mercury analysis. This method does not distinguish between the reaction of p-mercuribenzoate with thiol groups and the binding of the reagent to other sites within the protein molecule. The studies of Smalt *et al.* (45) on the inhibition of enzymes devoid of thiol groups by phenylmercury compounds may be cited in this connection. The results of Finkle and Smith (15) may be probably best explained on the basis of slow cleavage of disulfide bonds by the mercurial and perhaps binding to the imidazole groups of the two histidine residues in papain.

Papain activated by passage through the reducing column (15), contains 0.5-1.0 moles -SH per mole enzyme as determined spectrophotometrically with PCMB and NEM. This finding confirms the value reported for column-reduced papain by Finkle and Smith (15). Denaturation by a variety of methods did not affect the -SH titer. The difference in -SH content between the column-reduced enzyme and unactivated papain thus appears to be less than one -SH group per mole, regardless of whether the native or denatured protein is considered.

In agreement with the observations of Finkle and Smith (15), it was observed that the reaction with p-mercuribenzoate was very rapid, less than ten minutes being sufficient for complete reaction.

The application of the disulfide interchange reaction to papain leads to several significant conclusions. The results obtained with the interchange reaction indicate that there are eight groups capable of participating in this reaction per mole of papain. This result conclusively demonstrates the organic nature of the residues containing the eight sulfur atoms of papain. The specificity of the interchange reaction limits the possible forms in which the sulfur may be present to the following: R.S.S.R', R.SH, R.SOH, R.SO.S.R', $R.SO_2.S.R'$, R.S.CO.R', and any other forms which on exposure to concentrated hydrochloric acid at 39° would undergo a transformation (or dismutation) to one or more of the compounds listed above. However, sulfate esters, sulfonic acids, and all inorganic forms of sulfur are ruled out.

Since the behavior of papain in the exchange reaction was very similar to that of other proteins of known disulfide and sulfhydryl content, the results obtained are consistent with the assumption that the eight sulfur atoms in papain are present in the form of disulfide and sulfhydryl groups.

As discussed above, only a fraction of a sulfhydryl group has been found in unactivated papain. Hence, it would appear that the eight sulfur atoms in papain are present in the form of disulfide bonds.

Argentimetric amperometric titrations in presence of sulfite were used to test this hypothesis. It was found that three of the four disulfide bridges of papain can be readily cleaved by sulfite, in the absence of denaturing agents, at near-neutral pH. Sodium lauryl sulfate and 8M urea do not appear to denature papain sufficiently at near-neutral pH to permit cleavage of the fourth disulfide bond, and the titer in these denaturing agents is essentially the same as that obtained with native papain. Urea is known to be a poor denaturing agent for papain. Acid on

the other hand, is known to denature papain extensively and irreversibly (144). When papain was first denatured with acid and then brought to near-neutral pH with 8M urea solution, all the four disulfide bonds in papain could be readily titrated. The difference in reactivity towards sulfite found between the first three disulfide bonds in papain and the fourth is analogous to that found in insulin where two of the disulfide bonds are readily cleaved by sulfite in the native protein, while cleavage of the remaining bond requires denaturation (55). Similar observations have been made in the case of several other proteins (82,132).

The possibility was next investigated that the eight sulfur atoms could be accounted for on the basis of the half-cystine content of the protein. It was felt that the low yield of cysteic acid obtained in previous studies (68,69) could perhaps be explained by the observation mentioned above that one of the four disulfide bridges appears to be present in an exceedingly tightly coiled portion of the molecule and may be essentially unavailable to oxidation under the conditions usually employed.

This possibility was examined by carrying out the performic acid oxidation of acid-denatured, sulfite-treated papain, in which the secondary structure has been subjected to severe disorganization.

The uncorrected values for the cysteic acid and half-cystine obtained on amino acid analysis of acid hydrolysates of the oxidized material are 6.2 moles of cysteic acid and 0.8 moles of half-cystine per mole of papain.

The yield of 7.7 moles of cysteic acid per mole of papain obtained after acid hydrolysis of papain oxidized by the modified procedure of Moore indicates clearly that the sulfur content of papain may be accounted for completely in terms of the half-cystine content of this

protein.

The low yield of cysteic acid obtained in previous studies (68,69) was probably due to losses resulting from over-oxidation.

The information derived from the studies reported above still does not explain satisfactorily the mechanism of activation of papain. From the observation that native, unactivated papain contains in essence no sulfhydryl while the activated papain molecule contains one mole of sulfhydryl per mole of enzyme, it would appear on superficial examination that activation involves the cleavage of a disulfide bond. The finding of one thiol group in papain activated by cyanide or sulfite could easily be explained by reactions such as (1) and (2):

$$R\text{-}S\text{-}S\text{-}R' + SO_3^{=} \rightleftharpoons R\text{-}S\text{-}SO_3^{=} + R'S^{-} \quad (1)$$

$$R\text{-}S\text{-}S\text{-}R' + CN^{-} \rightleftharpoons R\text{-}S\text{-}CN + R'S^{-} \quad (2)$$

R'SH being the thiol group formed by activation.

This explanation is not satisfactory in the case of column-reduced papain. Sulfur analysis of this material indicates that no thioglycolate is transferred from the column to the protein on activation, i.e., no reaction of the type

$$R\text{-}S\text{-}S\text{-}R' + HS\text{-}CH_2COOH \rightleftharpoons R\text{-}S\text{-}S\text{-}CH_2COOH + RSH \quad (3)$$

takes place, but rather it is more likely that an actual reduction occurs

$$R\text{-}S\text{-}S\text{-}R' \rightleftharpoons RSH + R'SH \quad (4)$$

If it is assumed that equation 4 represents the true state of affairs, it would be expected that column-reduced papain should have two thiol groups per mole of the active enzyme. This is apparently not the case - only one

group per mole of the enzyme can be detected with PCMB or NEM. Two rather unconvincing explanations may be put forward to account for this finding. The first possibility is that either one or both of the thiol groups produced on activation may be extremely labile and oxidize very rapidly in the absence of thiol compounds and/or metal chelating agents. Another possibility is that one of the thiol groups formed may participate in an intramolecular interaction with some side-chain in the protein, thus forming the "active site". Such an interaction could perhaps so alter the reactivity of the thiol group, both from the viewpoint of chemical reactivity and perhaps also from the aspect of steric accessibility, that it would no longer react with the usual sulfhydryl reagents. This possibility does not appear very likely since the thiol titer of the column-reduced enzyme is not affected by denaturation.

In conclusion, while the sulfur content of papain can be accounted for satisfactorily as consisting of eight half-cystine residues, this does not provide a satisfactory answer to the problem of activation. This problem is considered more fully in the Concluding Discussion to this Thesis.

CHAPTER IV.

ULTRAVIOLET DIFFERENCE SPECTRA OF PROTEINS

INTRODUCTION

As discussed in Chapter I, Smith (22) has proposed on the basis of thermodynamic, kinetic, and chemical considerations that the reactive group at the active site of papain involves a thiol ester which is formed and maintained by the folding energy of the protein. It was felt that a proof of the presence of such a bond in papain could be obtained by employing the technique of difference spectroscopy in the ultraviolet region.

A Survey of the Literature on the Ultraviolet Difference Spectra of Proteins

The ultraviolet spectra of proteins have been extensively studied in the region 260 -320 mμ. In this region, the protein absorption may be accounted for almost entirely on the basis of their tryptophan and tyrosine content (145). The absorption due to the peptide bond has been shown to be only 5 per cent of the total absorption at 253.7 mμ, and to be negligible at about 280 mμ (148). Investigations at lower wavelengths have been hampered by the high absorbancy of proteins below 250 mμ. It has been reported (146) that the peptide bond has an absorption band with a $\lambda_{max.}$ in the region of 185 mμ.

It has frequently been observed that the absorption maxima of proteins at wavelengths longer than 250 mμ are displaced towards the red as compared to mixtures of free amino acids which duplicate the composition of the protein in question (147,148). It is possible to identify the vibrational fine structure bands of the aromatic chromophores in proteins (149-151). On comparison of these with the corresponding absorption bands of the free amino acids, it is evident that the entire absorption of the

aromatic groups has been shifted towards the red in many proteins, the magnitude of the shift varying from one protein to another and, to a certain extent, also differing from band to band in a single protein. Beaven et al. (152) have shown that the presence of the peptide bond accounts for spectral displacements of the aromatic chromophore bands of no more than 5-10 A.

In their classical spectrophotometric study of the ionization of tyrosine in egg albumin and insulin, Crammer and Neuberger (153) were able to show that the complete ionization of tyrosine in egg albumin was achieved only after denaturation of the protein. They estimated the pK of the phenolic hydroxyl groups in denatured ovalbumin to lie between 11.4 and 11.8. Crammer and Neuberger (153) pointed out that intramolecular hydrogen bonding in salicylic acid exerted a profound effect on the pK values of the carboxyl and phenolic -OH groups of this compound. They suggested that tyrosine -OH groups in native proteins may be involved in an analogous type of bonding, the carboxylate ion donor being another amino acid residue, and that this bonding could account for the observed spectral shifts.

Kauzmann (154) pointed out that non-polar side-chains of a protein molecule extending into an aqueous medium interfere with the hydrogen bonding between water molecules, i.e., they make "holes" in the structure of water with a resulting free energy increase. These non-polar residues would tend to aggregate in the interior of the molecule and form "hydrophobic" regions. Tanford et al.(155) concluded from their study of phenolic hydroxyl ionization in ribonuclease that three of these residues were involved in internal bonding of considerable strength. Tanford et al. (155) showed that the change in environment of the abnormal phenolic groups in ribonuclease was far greater than could be accounted for by simple hydrogen bonding and suggested that the inclusion of these residues within hydrophobic regions provided a more satisfactory explanation of the results.

The use of difference spectra for the study of changes in the ultraviolet spectra of proteins on proteolysis or denaturation was introduced by Laskowski et al. (156) in 1956. If the molar extinction coefficients, ϵ_1 and ϵ_2 of a given molecule in two different states, 1 and 2, are compared directly as a function of wavelength, the resulting plot of $\Delta\epsilon$ versus λ, where $\Delta\epsilon = \epsilon_2 - \epsilon_1$, is designated as a difference spectrum.

Laskowski et al. (156) and Scheraga (157) titrated insulin and ribonuclease, respectively, with acid and measured the $\Delta\epsilon$ between the acid and neutral solutions over the wavelength range 270-300 mμ. A bathochromic shift was observed in both cases and a difference spectrum with minima at about 280 mμ and 287 mμ was obtained. A similar spectrum was obtained on tryptic digestion of insulin (156). By plotting $\Delta\epsilon_{287}$ as a function of pH, Laskowski et al. (156) and Scheraga (157) showed that it increased in the pH range from 4 to 2 for insulin and 2 to 1 for ribonuclease. On the basis of these findings, they suggested that tyrosyl-carboxylate bonds were present in native proteins and that these bonds were broken on lowering the pH because of the neutralization of the carboxylate group involved. Evidence specifically supporting the carboxylate group as involved in tyrosyl-carboxylate bonding in proteins had been previously put forward by Harrington (158) on the basis of his studies on the liberation of acid- and base-binding groups during denaturation of ovalbumin by guanidinium chloride.

From a study of the effects of sodium acetate on the aqueous solubility of tyrosine, Wetlaufer (159) concluded that the probable free energy of formation of tyrosyl-acetate hydrogen bond was approximately 1400 cal./mole, i.e., of the same order as the free energy of peptide-like hydrogen bonding calculated by Schellman (160).

The view of Laskowski et al.(156) that the difference spectra arose

as a result of changes in the hydrogen-bonding of tyrosyl groups in proteins gained wide acceptance (161-165). Recent work by Wetlaufer *et al.* (166), Donovan *et al.* (167) and Bigelow and Geschwind (168,169) has cast some doubt on the validity of the above interpretation.

Wetlaufer, Edsall and Hollingworth (166) showed that in solutions of tyrosine, O-methyltyrosine and glycyl-O-methyltyrosine, the ionization of an adjacent group gave rise to difference spectra similar to those observed in proteins on lowering the pH. Wetlaufer *et al.* (166) pointed out that for spectral changes in these compounds, mechanisms involving intermolecular hydrogen bonding are ruled out because the effect was found to be concentration independent and that intramolecular hydrogen bonding was ruled out on steric grounds. Concentrated solutions of urea and of sodium acetate also produced difference spectra with both tyrosine and O-methyltyrosine with great similarity to those resulting from the ionization of a group within the molecule. From these findings, it was concluded that the difference spectra could be produced by dipole-dipole interactions, general ion-dipole interaction, or specific hydrogen bonding in the case of tyrosine. The difference spectra obtained from glycyl-O-methyltyrosine suggested that the ionizing group must be no further than about 10 A. away from the chromophore. The study of Wetlaufer *et al.* (166), while not invalidating the postulated tyrosyl-carboxylate interactions, served to emphasize the variety and complexity of the factors which could contribute to the observed phenomena. Donovan *et al.*(167) reported a similar study on tryptophan. Many workers (167,170-172) found blue shifts of the absorption band of this chromophore in the investigation of the difference spectra of a number of proteins on denaturation. These shifts cannot be explained by changes in hydrogen bonding of these residues and the suggestion has been made that the

non-polar chromophores take part in hydrophobic bonding (172,173,155).

In an important study, Bigelow and Geschwind (168) investigated the effect of media of high refractive index on the spectra of a number of model compounds: tryptophan, tyrosine, phenylalanine, indole, phenol and O-methyl tyrosine. Bigelow and Geschwind (168) found a distinct shift in the spectra of the above compounds to longer wavelengths, the red shift being a function of the refractive index of the solvent. Thus, solutions of sucrose and urea adjusted to the same refractive index brought about very similar red shifts. A relation between the spectral shift and the index of refraction of the solvent had been reported previous to this work (174-176). It has been suggested that the dependence upon the refractive index of the solvent is apparently due to the dielectric constant or the dipole moment of the solvent which affects the absorption bands and the refractive index in the same way.

Bigelow and Geschwind (168) pointed out a further complication in the interpretation of the tyrosine difference spectra. In low urea concentrations, ribonuclease (168), chymotrypsinogen (170) and bovine serum albumin (173) showed urea-induced red shifts. Thus, at non-denaturing concentrations, the effect of urea on these proteins is similar to its effect on tyrosine at any concentration. This indicates that in the three proteins mentioned above, some of the tyrosine residues are on the surface of the protein and behave like normal tyrosine residues. In all of the three cases denaturation at higher urea concentration is accompanied by the characteristic blue shift.

In view of the above observations, it has been suggested (168) that some aromatic residues in native proteins are in a medium of high refractive index, and that upon denaturation some of these become exposed to the medium which has a lower refractive index. If refractive index changes are the

overriding factor in determining the spectral shifts discussed above, a blue shift would be expected in going from a medium of high refractive index to one of low refractive index. Further, the denaturation blue shift observed on the urea denaturation of proteins must represent the difference between the blue shift due to the change in refractive index of the environment of the aromatic chromophores and the red shift which is characteristic of the effect of urea on normal tyrosine or tryptophan residues.

Bigelow (169), taking the above considerations into account, has been able to show that the spectral changes occurring on the urea denaturation of ribonuclease could be interpreted in terms of refractive index changes. He found no evidence that tyrosyl-carboxylate hydrogen bonds make any contribution to the observed denaturation blue shift.

To summarize the salient points of the preceding discussion:

(1) When denaturation makes an anomalous residue normal, a "denaturation blue shift" in the spectrum of the protein is observed.

(2) If the denaturation is carried out under conditions which do not affect the spectrum of normal aromatic chromophores, the amount of the denaturation blue shift is obtained directly from the observed difference spectrum.

(3) If the denaturing agent does affect the spectrum of normal aromatic residues the appropriate correction must be applied to the observed change to obtain the denaturation blue shift.

(4) The observed difference spectra may be due to changes in the hydrophobic or electrostatic environment of the chromophoric residue. No simple evaluation of the relative contributions of dipole-dipole, ion-dipole, hydrogen-bonding and environment factors is as yet possible.

The study of difference spectra has proved extremely fruitful in detecting minute changes in native protein structure (157,162,165). Attempts have been made to correlate known primary structure of proteins with the observed changes in the ultraviolet absorption spectra (177). It is hoped that ultimately this approach will give insight into those parts of the structure of proteins which involve chromophoric groups which can be studied by the technique of ultraviolet difference spectroscopy.

EXPERIMENTAL

Materials and Methods

The proteins, amino acids and the other compounds the spectra of which were investigated are listed in Table IX together with the source and the extinction coefficient, wherever the latter was used for the determination of concentration.

All solutions were made up in deionized, glass-distilled water. Since both cations and anions have been shown to distort difference spectra of proteins under a variety of conditions (164,173) the use of buffers was avoided entirely. Adjustment of pH was carried out with 1N HCl and 0.2 N NaOH. The Radiometer Type TTT1a Autotitrator was used for the measurement of pH.

All the spectra were obtained with the Cary Model 14 Recording Spectrophotometer. Square cells with 10 mm light-path with special fused silica windows for high transmittance in the short ultraviolet region were used. The stray light correction was found to be negligible over the wavelength range and optical density range investigated.

Unless otherwise specified, all solutions were scanned within 20 min. of mixing. The methods of denaturation generally employed were acid

denaturation, alkali denaturation, heat denaturation, or a combination of two or more of these techniques. In addition, the effect of sodium lauryl sulfate and concentrated urea at near neutral pH on ovalbumin, and the autolysis of pepsin were also examined.

It may be briefly stated that all of the above mentioned methods of denaturation resulted in the appearance of the usual tyrosine and tryptophan difference peaks in the 278-295 mμ region as well as of a more prominent peak at 230-235 mμ.

RESULTS

Proportionality of the difference spectrum in the region 230-235 mμ to protein concentration.

The study of difference spectra in the far ultraviolet region offers many traps to the unwary. By far the greatest error in difference spectrophotometry is an instrumental error which may be encountered in solutions of high absorbancy (178,179). Such error is due to the fact that every monochromator gives, in addition to the selected wavelengths, a certain amount of light of random wavelengths, the so-called "stray light". It is possible to obtain valid measurements of high optical densities if the light is sufficiently monochromatic, i.e., the solution to the problem of stray light lies in reducing the proportion of stray light to selected light. The amount of stray light given is an intrinsic property of each monochromator.

Using the Beckman DU spectrophotometer with a photomultiplier, Mehler (178) and Fridovitch et al.(179) showed that, working with solutions of high absorbancy, artifacts represented by changes in molar extinction with concentration, shifts in the absorption maxima and appearance of artifactual maxima and minima in the difference spectra were observed.

TABLE IX

SUBSTANCES USED FOR SPECTROPHOTOMETRIC STUDIES

A. PROTEIN	SOURCE	$\lambda_{max.}$	$E^{1\%}_{1\ cm.}$	Ref.
Chymotrypsinogen	Armour Lot R337254	280	20.0	(70)
Insulin	Squibb, Zn-free	280	10.0	(118)
β-Lactoglobulin	3xcryst.	280	9.5	(18)
Lysozyme	Armour Lot 003L1	282	27.3	(122)
Ovalbumin	Nutritional Bioch. Corp., 2xcryst., salt-free	280	7.35	(120)
Papain	3xcryst.	278	25.0	*
Pepsin	3xcryst.	280	14.3	(125)
Protamine sulfate	Squibb	-	-	-
Ribonuclease	Worthington Bioch. Corp. Lot 558	280	6.9	(116)
Serum albumin (bovine)	Armour Lot 128-176	280	6.6	(114)
Serum albumin (human)		280	5.3	(83)
B. AMINO ACIDS		$\lambda_{(m\mu)}$	ϵ_M	
L-arginine	2xcryst.	205	1.35×10^3	(186)
L-glutamic acid	2xcryst.	-	-	-
L-histidine monoHCl	Merck	205	5.2×10^3	(186)
L-methionine	1xcryst.	-	-	-
L-phenylalanine	2xcryst.	257.5	1.95×10^2	(145)
L-tryptophan	2xcryst.	278	5.55×10^3	(145)
L-tyrosine	2xcryst.	274.5	1.34×10^3	(145)
C. OTHER COMPOUNDS				
Urea	Mallincrodt (recryst.)	-	-	-
L-homocysteine thiolactone	Calif. Foundation for Bioch. Research	237.5	4.34×10^3	*
Benzoylglycine thiol ethyl ester		235	15.5×10^3	*
Thiolhistidine	Calif. Foundation for Bioch. Research	257.5 208	2.00×10^4 9.3×10^3	* *
Glutathione	Schwartz	-	-	-
Poly-L-glutamic acid	Gift of Dr. Katchalski	-	-	-

* $E^{1\%}_{1\ cm}$ determined in the course of the present investigation.

On the other hand, using the Cary spectrophotometer, which has a double beam monochromator, both Fridovitch et al. (179) and Mehler (178) reported that accurate measurements could be made on solutions with background optical densities exceeding 5.

The background optical density at which serious errors are obtained is a function not only of the light source, monochromator and phototube, but also of the wavelength selected and the spectrum of the substance studied. It is necessary, therefore, to test each instrument with the type of substance studied over the background absorbancy range desired and the required wavelengths in order to determine the conditions permitting accurate measurement.

A simple and sensitive test for the absence of stray light artifacts is the adherence to Beer's law at the required wavelength and over the background absorbancy range desired.

It was found that at background absorbancy values at 230 mμ of 4.6 or less, all of the compounds studied gave linear $\Delta D(230\text{-}235)$ versus concentration plots. Representative results for human serum albumin are given in Table X. In all of the studies reported below, the background absorbancy was less than 4.6.

Effect of pH on the Ultraviolet Spectra of Proteins

Examination of the difference spectrum obtained by comparing papain at neutral pH with papain in acid solution (pH 1.4) showed peaks at 234 mμ and at 287.5 mμ. The 287.5 mμ peak could be accounted for as arising from the alteration in the environment of the numerous tyrosine residues in papain resulting from denaturation. The 234 mμ peak is nearly at the expected position for the disruption of an aliphatic thiol ester, 231 mμ, or of a hydrogen-bonded -SH group, 235 mμ (180). While the observations are in a

TABLE X

CONCENTRATION DEPENDENCE OF DIFFERENCE ULTRAVIOLET SPECTRUM OF HUMAN SERUM ALBUMIN AT 234 mμ.

(pH 1.4 vs. pH 5.2)

Human serum albumin conc., c, mg./ml.	ΔD_{234}	$\Delta D_{234}/c$
0.096	0.034	0.35
0.30	0.084	0.28
0.61	0.167	0.27
0.95	0.261	0.27
1.05	0.293	0.28
1.57	0.355	0.23

qualitative agreement with the concept of a cleavage of a thiol ester associated with denaturation, the quantitative correlation with this view is not obtained. The $\Delta\epsilon_M$ at 287.5 mμ is approximately 2,400, indicating that 12-16 tyrosine side-chains exhibit "disturbed" spectroscopic behavior (166). The $\Delta\epsilon_M$ at 234 mμ is 11,450. The cleavage of a thiol ester would be expected, at most, to result in a $\Delta\epsilon_M$ change of 4,500. The observed value if interpreted solely on the basis of cleavage of thiol esters would call for the presence of at least three such groups in papain. This supposition is untenable in view of the data already presented on the nature of the sulfur atoms in papain. Hence, another reason for the appearance of the difference peak at 234 mμ must be sought, although , of course, some measure of contribution to the observed peak from a bond of the thiol ester variety may be included in the observed value. It may be noted in passing that the acetyl-imidazole grouping exhibits its absorption maximum at 245 mμ (181) and can be excluded on this basis.

To examine the hypothesis that the observed difference peak at 234 mμ is a reflection of a change in protein conformation on denaturation, the effect of acid on a number of proteins was examined. The results are presented in Table XI.

From this investigation, it was immediately apparent that proteins which are known to undergo structural changes in acid solution (human serum albumin, bovine serum albumin, ribonuclease) all showed the difference peak in the wavelength range 230-235 mμ on comparing acid with neutral solutions (Figure 11). On the other hand, proteins known to be stable in acid solution, such as β-lactoglobulin, pepsin, chymotrypsinogen did not show the spectral shift in the far ultraviolet unless a more drastic denaturation treatment

than mere acidification was employed. It is noteworthy, that the peaks in the 278-295 mμ range attributable to tyrosine and tryptophan appear only under conditions which result in the appearance of the 230-235 mμ difference peak. In no case was the 230-235 mμ peak observed without the concomitant appearance of the tyrosine and tryptophan peaks.

Combination of Heat with Alkali and Acid Denaturation

As mentioned above, mere lowering of the pH was insufficient to produce the difference spectrum in the case of β-lactoglobulin, chymotrypsinogen and lysozyme. These proteins are known to be stable in acid solution.

When alkaline denaturation was employed, in conjunction with heating in some cases, and acidification of the alkaline protein solution, the comparison of the denatured with the native protein at neutral pH, in each case resulted in the appearance of the characteristic difference spectrum as shown in Fig. 12. The results and the conditions employed are listed in Table XI.

It appears, therefore, that the hypothesis that the difference peak in the 230-235 mμ region is associated with changes in protein conformation on denaturation is correct.

Effect of Other Denaturing Agents

Since the above experiments were all carried out by comparing the neutral with the acid solution of the proteins studied, the criticism could be made that the observed difference peak was due to protonation of groups inaccessible in the native protein. For example, carboxyl groups with highly abnormal pK values have been observed in a large number of proteins (182,183). The un-ionized carboxyl group absorbs less strongly at 225 mμ than the $-COO^-$ species (187), and it could be argued that protonation

of a large number of masked ionized carboxyl groups on denaturation could make a contribution to the difference peak at 230-235 mμ. It was desirable, therefore, to use methods of denaturation which would involve no change in pH, i.e., the native and denatured proteins could then be examined at the same pH.

The effect of denaturing agents at near-neutral pH was studied with ovalbumin. Sodium lauryl sulfate and urea were employed as the denaturing agents.

The results obtained are listed in Table XI. It will immediately be seen that these denaturing agents produced the same qualitative changes in the far ultraviolet spectrum of ovalbumin as did the acid denaturation of the proteins discussed above.

The urea denaturation of ovalbumin is a comparatively slow reaction (154) whose rate is strongly dependent on the urea concentration and pH. By choosing the correct combination of these two variables, pH 7.8 in 0.012 M phosphate buffer, and 8 M urea, it was possible to investigate the rate of change of absorbancy at 232 mμ and 287 mμ. The change in the 232 mμ peak continued for a considerable time after the cessation of changes at 287 mμ. The $\Delta\epsilon_M$ (233 mμ) changed from a value of 38,600 at 7 hours, to a value of 55,240 at 24 hours. During this time, the $\Delta\epsilon_M$ values at 280, 287, and 293 mμ did not change.

It may be concluded that the change in absorbancy at 230-235 mμ represents a change in the environment of a chromophoric group or groups distinct from either tyrosine or tryptophan side-chains. Some contribution to the 230-235 mμ difference peak may be made by changes in the tyrosine and tryptophan absorption, but inspection of the results obtained with a large number of proteins (Table XI) clearly shows that absolutely no correlation

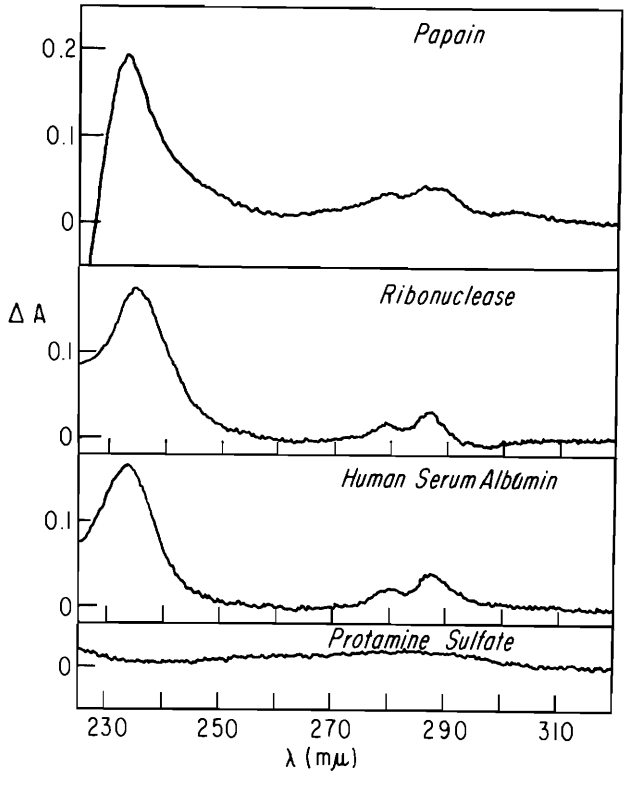

Fig. 11. Effect of acidification on the ultraviolet difference spectra of proteins. Papain (0.034%) at pH 5.2 vs. pH 1.4; human serum albumin (0.049%) at pH 5.2 vs. pH 1.4; ribonuclease (0.086%) at pH 5.4 vs. pH 1.4; protamine sulfate (0.016%) pH 5.2 vs. pH 1.5. Light path: 1 cm.

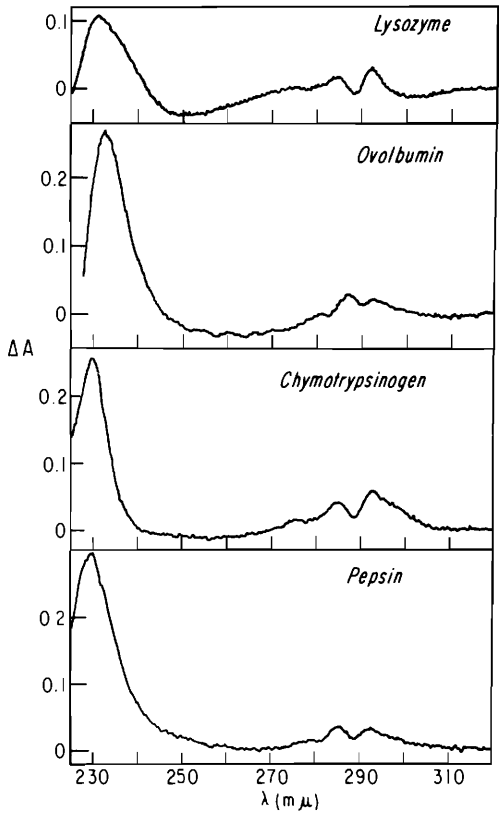

Fig. 12. Effect of alteration of primary and secondary structure on the ultraviolet difference spectra of proteins. Lysozyme (0.013%) at pH 5.4 vs. lysozyme heated at 92° and pH 11.4 for 10 min., cooled, and adjusted to pH 1.4 prior to scanning. Ovalbumin (0.046%) at pH 7.8 vs. ovalbumin in 8M urea at pH 7.8 after 24 hours at 25° (uncorrected for urea absorption). Chymotrypsinogen (0.037%) at pH 6.0 vs. chymotrypsinogen exposed to pH 11.5 at 25° for 10 min., then acidified to pH 1.4 and allowed to stand for 10 min. prior to scanning. Pepsin (0.038%) at pH 4.6 vs. pepsin at pH 1.4, both solutions allowed to stand at 4° for 2 hours prior to scanning. Light path: 1 cm.

TABLE XI

DIFFERENCE SPECTRA FOR THE PROTEIN IN NEUTRAL SOLUTION AND THE PROTEIN UNDER THE CONDITIONS SPECIFIED

Protein	Denaturation Conditions	$\lambda_{max.}$ Å	ϵ_M Native Protein	$\Delta\epsilon_M$
Bovine Serum Albumin (pH 6.0)	pH 1.4, room temp.	2880	27,700	7,220
		2790	42,400	3,600
		2340	194,300	31,050
Human Serum Albumin (pH 5.2)	pH 1.4, 25°	2880		7,530
		2795		3,030
		2330		29,250
Insulin (pH 4.0)	pH 1.5, 10 min., 25°	2340		2,400
Papain (pH 6.0)	pH 1.4, 10 min., 25°	2350	103,000	11,450
		2875	34,300	2,420
Ribonuclease (pH 5.4)	pH 1.4, 25°	2875		520
		2790		310
		2350		3,040
β-Lactoglobulin (pH 6.0)	pH 1.4, 24 hours, 25°*	2365		1,230
	Alkaline denaturation*	2365		12,250
Lysozyme (pH 6.0)	pH 1.4, 39°, 24 hours*	none		-
	Alkaline denaturation*	2928		3,130
		2860		1,670
		2315		11,280
	Acid Denaturation***	2925		1,040
		2850		1,050
		2305		4,160
		2270		6,580
	pH 5.2, 20 min., 92°	none		-
Chymotrypsinogen (pH 6.0)	pH 1.4, room temp.	none		-
	Alkaline denaturation*	2930	36,600	6,290
		2855	45,600	6,300
		2760	47,500	130
		2305	230,600	27,280
	Alkaline denaturation**	2928	36,620	3,910
		2855	45,600	2,780
		2300	230,650	16,930
		2260		1,000
	Acid denaturation***	2930		4,300
		2855		3,250
		2760		1,190
		2300		18,600

TABLE XI (cont.)

Protein	Denaturation Conditions	$\lambda_{max.}$ Å	ϵ_M Native Protein	$\Delta\epsilon_M$
		(2300)		
Protamine sulfate (pH 5.0)	pH 1.4, 39°, 2 hours	none	approx. 11,000	-
Ovalbumin (pH 6.5)	pH 6.5, 1% sodium lauryl sulfate. Scanned 30 sec. after mixing.	2930	15,790	2,40
		2870	27,920	2,00
		2320	214,200	27,45
Ovalbumin (pH 7.8)	pH 7.8, 0.012M phosphate buffer, 8M urea, 24 hours at 24°.	2930		2,87
		2870		3,96
		2800		1,04
		2330		55,24

* Alkaline denaturation: 10 min. at pH 11.4 in a water bath at 92°, followed by acidification to pH 1.4. Allowed to cool for 10 min. prior to scanning.

** Alkaline denaturation: 10 min. at pH 11.4 at 25° followed by acidification to pH 1.4. Allowed to stand at pH 1.4 for 10 min. prior to scanning.

*** Acid denaturation: Heated for 10 min. at pH 1.4 at 92°. Allowed to cool for 10 min. prior to scanning.

\+ This value was obtained after correction for urea absorption at 233 mμ.

exists between the tyrosine and tryptophan content of the proteins investigated and the magnitude of the 230-235 mμ difference peak obtained on denaturation.

Effect of Autolysis

It was of interest to know whether changes in primary structure brought about by proteolysis would also result in the appearance of the difference peak in the 230-235 mμ region. Proteolysis is known to be accompanied by the appearance of the tyrosine and tryptophan difference spectra (165,170,171).

This point was examined by studying the difference spectra obtained by comparing pepsin at a given pH with its autolysate at different periods

of time. This experiment was carried out at two different temperatures and two different pH values. The difference spectrum showed a peak at 230 mμ, the peaks due to tyrosine at 279 and 286 mμ, and the peak due to tryptophan at 292 mμ (see Figure 12).

A reasonably good correlation was obtained between the $\Delta\epsilon_M$ at 230 mμ and the amount of α-amino nitrogen as determined by the ninhydrin method (184). Thus, at pH 1.45, a $\Delta\epsilon_M$ decrease of 13,360 was obtained following 2 hours proteolysis at 39° and a ninhydrin color increment per mole (ΔN_{molar}) of 18,900, giving $\Delta\epsilon_M/\Delta N_{molar} = 0.71$ while at pH 4.5 the decrease at 230 mμ $\Delta\epsilon_M = 9{,}000$ and $\Delta N_{molar} = 11{,}500$ giving $\Delta\epsilon_M/\Delta N_{molar} = 0.78$.

Difference Spectra of the Amino Acids

Since acid denaturation was employed in a large number of cases, it was desirable to check whether the change in pH brought about extensive changes in the spectra of the free amino acids themselves. This was especially important in the case of tyrosine, tryptophan and phenylalanine, since the spectra of these amino acids rise steeply at 240 mμ (145).

Table XII lists the values for the maxima and minima found in difference spectra obtained by comparing a neutral solution of each of the amino acids studied with an acid solution of the same amino acid. Clearly, the results do not explain, even qualitatively, the changes observed in ultraviolet spectra of proteins on denaturation.

The only two amino acids which show peaks in the difference spectrum in the region of 230 mμ are tyrosine and tryptophan. Tyrosine actually absorbs more strongly at 230 mμ in acid solution than in neutral solution.

While it is not possible to extend quantitative conclusions formed on the basis of changes in the spectra of free amino acids to changes in their behavior when involved in both a peptide linkage and interrelations with side-chains of other amino acids within the protein molecule, it may be

emphasized that in the case of the difference spectra of tyrosine and tryptophan, the changes observed with the free amino acids are qualitatively in the same direction and at the same wavelengths as those observed in the difference spectra of proteins on denaturation.

It may be noted that no difference peaks were observed on comparing an acid and a neutral solution of glutathione in the wavelength range 220-320 mμ.

The results in Table XII represent the first complete description of the difference spectra of the amino acids in the wavelength range 210-360 mμ. The difference spectra of tyrosine (166) and tryptophan (167) have been described previously. For these two amino acids, the values given in Table XII are in good agreement with those listed in the literature.

Donovan et al. (167) have listed the positions of the peaks in the difference spectrum of phenylalanine and these correspond closely to those listed in Table XII.

No attempt was made to check the molar extinction coefficients of tyrosine, tryptophan and phenylalanine. The values reported by Beaven and Holiday (145) were used. The molar extinction coefficients for tyrosine and tryptophan have been checked recently by Strickland et al.(185) and agree well with those given by Beaven and Holiday (145).

The values reported by Saidel, Goldfarb and Waldman (186) for the extinction coefficients of the amino acids at low wavelengths were in agreement with those obtained in the course of this work.

Difference Spectra of Thiol Esters and Thiolhistidine

The difference absorption spectra of L-homocysteine thiolactone, benzoyl glycine thiol ethyl ester and thiolhistidine were examined under a variety of pH conditions. The results are presented in Table XIII.

TABLE XII

MAXIMA AND MINIMA IN THE DIFFERENCE ABSORPTION SPECTRA OF THE AMINO ACIDS

Sample cell	Reference cell	$\lambda_{max.}$	ϵ_M(max.)	$\lambda_{min.}$	ϵ_M(min.
Tryptophan (pH 6.0	at pH 1.4	291.6 mμ	320	234 mμ	550
		284	140		
		225	2830		
Phenylalanine (pH 6.2)	at pH 1.35			222.5	145
		269	2	250	5
		265	2	256	7
		259	3	262	5
		252.5	1		
Tyrosine (pH 5.7)	at pH 1.4	285	40	232	590
		278	20		
Histidine (pH 4.0)	at pH 1.4	230	66		
	at pH 7.1	217.5	453		
	at pH 11.5			225	88

Note: $\lambda_{max.}$ and ϵ_M (max.) refer to peaks in the positive direction (i.e., those representing a higher absorbancy of the solution in the sample cell). $\lambda_{min.}$ and $\epsilon_{M(min.)}$ refer to peaks in the negative direction (i.e., those representing a higher absorbance of the solution in the reference cell).

Methionine showed an enhanced absorbance in acid solutions at wavelengths below 225 mμ. Arginine gave a flat difference spectrum over the wavelength range 220-320 mμ.

TABLE XIII

Maxima in the difference spectra of L-homocysteine thiolactone, glutathione, benzoylglycine thiol ethyl ester, urea and thiolhistidine, in the range 210-240 mμ.

Sample cell	Reference cell	$\lambda_{max.}$	$\Delta\epsilon_M$(max.)
L-homocysteine thiolactone (pH 6.0)	L-homocysteine thiolactone at pH 11.4 (following 2 hours at 39° at this pH)	237.5 mμ	4,300
Glutathione (pH 6.0)	Glutathione (pH 1.5)	None	-
Benzoylglycine thiol-ethyl ester (pH 6.0)	Benzoylglycine thiolethyl ester at pH 11.4 (following 2 hours at 39° at this pH)	235	14,000
Urea (10M) (pH 7)	Urea (10M) pH 1.4	218	0.56
Thiolhistidine (pH 5.2)	Thiolhistidine (pH 1.4)	None	None

L-homocysteine thiolactone was found to be extremely resistant to acid hydrolysis, only 6.6 per cent hydrolysis being observed after 24 hours at 39° and pH 1.4. Treatment with alkali at pH 11.4 for 2 hours resulted in almost complete hydrolysis of the thiolactone with the concomitant disappearance of the 237.5 mμ peak.

Benzoylglycine thiol ethyl ester was found to be resistant to acid. However, on prolonged standing at pH 1.4 and 39°, the spectrum altered with an overall increase in absorption and a shift of the $\lambda_{max.}$ from its original position at 235 mμ to 230 mμ. Alkali readily hydrolyzed this thiol ester.

Thiolhistidine gave flat difference spectra in the region 220-300 mμ, when the solutions of this compound at neutral pH were compared with those at acid pH values.

Difference Spectra of Poly-L-glutamic Acid

Consideration of the data obtained with the free amino acids and proteins indicated that an explanation for the appearance of the 230-235 mμ difference peak on protein denaturation would have to be sought elsewhere than in the interactions of the amino acid side-chains.

The possibility that the difference peak resulted from the alteration in the environment of the peptide backbone of proteins was next investigated.

Poly-L-glutamic acid was chosen as a model compound for the study of this possibility. Doty et al. (188) have shown that at pH 4, this molecule exists as an α-helix. As the pH is raised and the carboxyl group ionizes, repulsion between the adjacent $-COO^-$ groups results in a transformation of this helix into a random coil. Doty et al. (188) have shown that the proportion of helix to random coil follows very closely the ionization curve of the γ-carboxyl of glutamic acid.

Poly-L-glutamic acid thus offers a model system for the investigation of the effect of uncoiling of an α-helix on ultraviolet absorption.

On comparing a solution of poly-L-glutamic acid at pH 11.4 with a solution at the same concentration at pH 4.0, the difference spectrum showed a peak at 225 mμ. Further, the height of the 225 mμ peak was dependent on pH in the range 4-6 in an analogous fashion to that observed by Doty et al. (188) for the helical content (Figure 13).

Calculation of $\Delta\epsilon_{res.}$ at 225 mμ gave a value of 85. This value is somewhat lower than that observed for the proteins, e.g., approximately 170 for chymotrypsinogen and 120 for papain. Deuterium exchange studies on a number of the proteins used in this investigation (189) have indicated that the number of difficultly exchangeable hydrogens is comparatively low. This would indicate the absence of hydrogen bonding of the type observed in a

Pauling-Corey α-helix. On the other hand, little is known about the possible configurations in the so-called "random" sections of polypeptide chains in proteins where hydrogen-bonding could result in the formation of large ring structures. Such structures are known to absorb strongly in the far ultraviolet region. It is possible, therefore, that disruption of conformations other than helical could give rise to the observed changes.

In a recent investigation by ultraviolet absorption spectrophotometry of the helix-coil transition of poly-L-glutamic acid, Imahori and Tanaka (190) have shown that both forms have a maximum at 190 mμ, but that the coil absorbs much more strongly than the helix at this wavelength. The curves for the helix and the coil have an isosbestic point at approximately 220 mμ. This work indicates that on denaturation a very much larger peak may be expected in the difference spectrum in the 190-200 mμ region than that observed in the 230-235 mμ region.

Attempts to Detect a Thiol Ester in Papain

(i) Effect of Cyanide.

Freshly prepared cyanide solutions even at 0.4 M have negligible absorption in the region 225-320 mμ. It was possible, therefore, to compare the spectra of papain in the absence and presence of cyanide in this spectral region. The study of the sulfur distribution of papain indicated that the presence of a preformed thiol ester in the native, unactivated enzyme was unlikely. If activation does result in the formation of a thiol ester, a change in the absorption at 231-235 mμ would be expected on activation. When papain solutions in water were compared with those in 0.2 M cyanide at the same pH (7.8) and concentration, no peak could be detected in the difference spectrum in the range 220-320 mμ. The papain concentration used was such that the formation of 0.1 mole of thiol ester per mole of papain would have

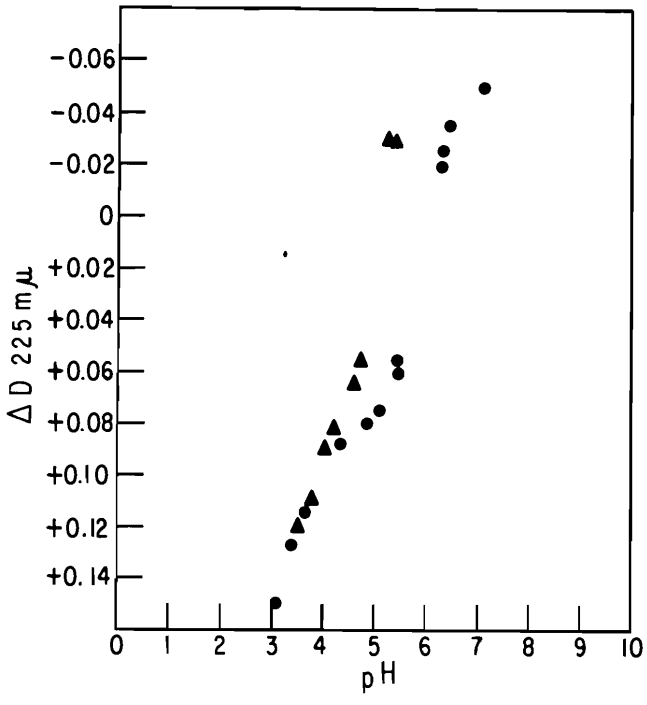

Fig. 13. pH dependence of the difference spectrum of poly-L-glutamic acid. Concentration of poly-L-glutamic acid = 0.22 mg/ml. Reference solution at pH 11, sample solutions at the pH indicated. ● in absence of salt, ▲ in 0.16 M NaCl.

been easily determined. The absence of the expected difference peak does not, of course, rule out the possibility that a thiol ester is, in fact, formed on activation. From the studies presented in preceding sections, it is clear that conformational changes have a profound effect on the ultraviolet spectrum in the 230-235 mμ region. It is possible that a conformational change occurring on activation obscures the change due to the formation of a thiol ester.

(ii) Effect of Hydroxylamine.

Incubation of papain with hydroxylamine (2 per cent) at pH 7.1, produced no change in the absorption in the 231-235 mμ region. This indicates that the presence of a preformed thiol ester in native, unactivated papain is unlikely.

It was not possible to examine the effect of a mixture of cyanide and hydroxylamine with papain, because of an interaction between these two compounds resulting in an enhancement of ultraviolet absorption in the region 200-240 mμ.

GENERAL DISCUSSION AND CONCLUSIONS

From the work presented in this Chapter, it appears well established that denaturation of proteins results in some conformational change which is associated with the appearance of a difference peak in the region 230-235 mμ.

Although the aromatic amino acids make a major contribution to the overall absorption of proteins in this region of the spectrum, a number of observations make it unlikely that the 230-235 mμ peak represents solely changes in the environment of the aromatic residues.

Firstly, there is no correlation between the extent of the 230-235 mμ peak and the tyrosine and tryptophan content of the proteins studied. There is also no correlation between the height of the 278-293 mμ peaks and the 230-235 mμ peak. Secondly, in the urea denaturation of ovalbumin, changes

at 233 mμ continue for a long time after cessation of changes in the 278-295 mμ region. Lastly, the difference spectra of the free amino acids offer no qualitative explanation of the observed changes.

The finding of a difference peak associated with the helix-coil transformation of poly-L-glutamic acid at 225 mμ indicates that backbone conformation changes are reflected in this region of the spectrum.

This gives support to the hypothesis that the change in the far ultraviolet spectra of proteins observed on denaturation is a reflection of changes in the conformation of the peptide backbone.

Since no correlation could be found between the observed difference peak and the helical content of proteins as determined by other methods, it is not as yet possible to define in structural terms the changes responsible for the observed spectral shift.

CHAPTER V.

PHENOLIC HYDROXYL IONIZATION IN PAPAIN

The difference spectra obtained by comparing papain in acid and neutral solution exhibit peaks at 287 and 279 mμ suggestive of the involvement of the phenolic -OH groups in hydrogen and/or hydrophobic bonding (156,166,168). The $\Delta\epsilon_M$ (287 mμ) was found to be 2420.

Wetlaufer et al. (166) have calculated from the data on a number of proteins that a $\Delta\epsilon_{res.}$ (287 mμ) of 150-200 is observed per tyrosine residue involved in hydrogen and/or hydrophobic bonding. From these values, it would appear that 12-16 phenolic hydroxyl groups in papain show "disturbed" spectroscopic behavior.

The results discussed in this section represent a further investigation of the behavior of the phenolic -OH groups in papain by means of spectrophotometric titration of this protein in the pH range 5.5-14.

Experimental.

Reagents.

Twice-recrystallized salt-free lyophilized papain was used in this study. Fresh stock solutions were made up each day and stored at 4°. The protein concentration in the stock solutions was determined from the light absorption at 278 mμ wavelength in an appropriately diluted solution. The value of $E^{1\%}_{1\ cm.}$ was found to be 25.0 (dry-weight, ash-free basis). The value for the molecular weight used in all the calculations was 20,700. A stock solution of sodium hydroxide was made by diluting a saturated solution of sodium hydroxide with carbon dioxide-free water. The concentration was checked by titration against standard potassium biniodate. The solutions for measurement were in the concentration range 1-2 x 10^{-5}M papain and their pH was adjusted by careful addition of appropriate amounts of acid or

base. Sodium chloride was used to bring the total ionic strength of each solution to either 0.01 or 0.2.

Measurements of pH.

Measurements of pH were made on a type TTT1a Autotitrator (Radiometer-Copenhagen) with a type C electrode. Correction for sodium ion errors was made with the aid of a nomograph supplied with the instrument. Solutions of high pH (in the range 12.5-14.0) were sodium hydroxide solutions of known molality containing sufficient sodium chloride to bring the ionic strength to 0.2. The pH of these solutions was computed from the activity coefficients obtained from hydrogen electrode measurements by Tanford (191). The Radiometer Autotitrator and the Cary Spectrophotometer (see below) were both placed in a room, the temperature of which was maintained at 27.0 $\pm$ 0.2°C. All the measurements reported below were performed at this temperature.

Ultraviolet Light Absorption Measurements.

Absorption measurements were made on a Cary Model 14 Recording Spectrophotometer. Cylindrical tightly-stoppered cells with a 10-mm light path were used throughout. The cells were matched with water over the range 400-220 mμ with the multipots on the instrument.

Results.

Ultraviolet Absorption Spectrum of Papain in Neutral and Alkaline Solution.

The ultraviolet absorption spectrum of papain in neutral solution, immediately on adjustment to pH 12, and immediately after adjustment to pH 13, are shown in Figure 14. It is immediately apparent on inspection of Figure 14, that not all the phenolic hydroxyl groups are ionized at pH 12. In fact, the situation appears to be very similar to that described by Crammer and Neuberger (153) for ovalbumin.

Time Dependence.

It was observed that the optical density of papain solutions in the pH range 12-12.5 changes with time. A typical plot of change in absorbancy vs. time is shown in Figure 15. The molar extinction at pH 12.2, for example, rises from about 40,000 (obtained within 30 seconds of adjusting the pH) to 51,000 ± 1,000 at the end of approximately 60 minutes at 27°. Adjustment of the pH of a solution from 6 to 13, is accompanied by an instantaneous change in the molar extinction from 12,500 to about 50,000. A slow increase in absorbancy at 295 mμ is then observed, a final value of 52,500 being reached in about 4 hours at 27°. Exposure to normal sodium hydroxide (near pH 14) for 10-20 min. at 39° results in a final molar extinction of 52,800 at 295 mμ.

It was important to decide whether the time-dependent changes at 295 mμ observed at pH values greater than 12 were due to slow ionization of a few phenolic hydroxyl groups, or, whether they were due to some other cause such as an increase in light scattering caused by denaturation and aggregation.

The ionization of the phenolic hydroxyl groups in tyrosine (153), polytyrosine (192), and in a number of proteins (153,155) is accompanied by a large increase in extinction at 295 mμ, a considerably smaller change at 265 mμ, and a very small change at 278 mμ.

The wavelength dependence of the increase observed with papain follows that of tyrosine and thus appears to be due to a slow ionization of phenolic hydroxyl groups.

A similar situation exists in ribonuclease (155). In this protein a slow ionization of three phenolic hydroxyl groups takes place at pH values greater than 12, the wavelength dependence of the process being

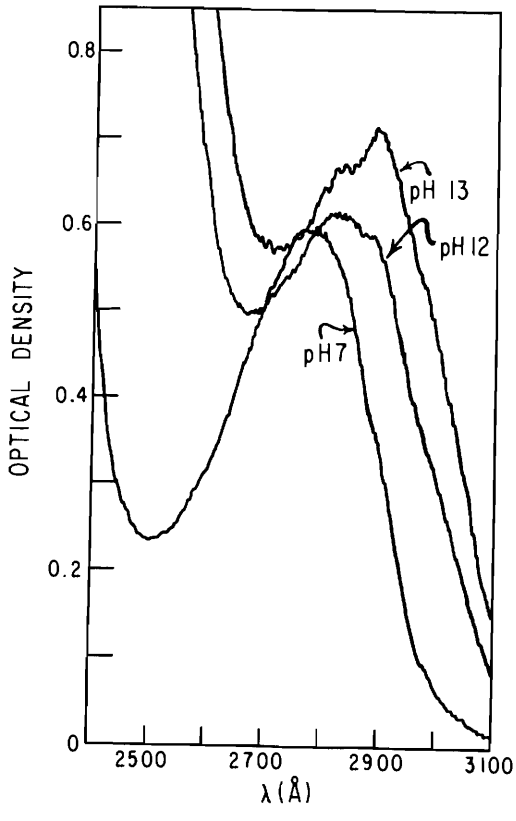

Fig. 14. Ultraviolet absorption spectrum of papain as a function of pH. Spectra scanned immediately after pH adjustment.

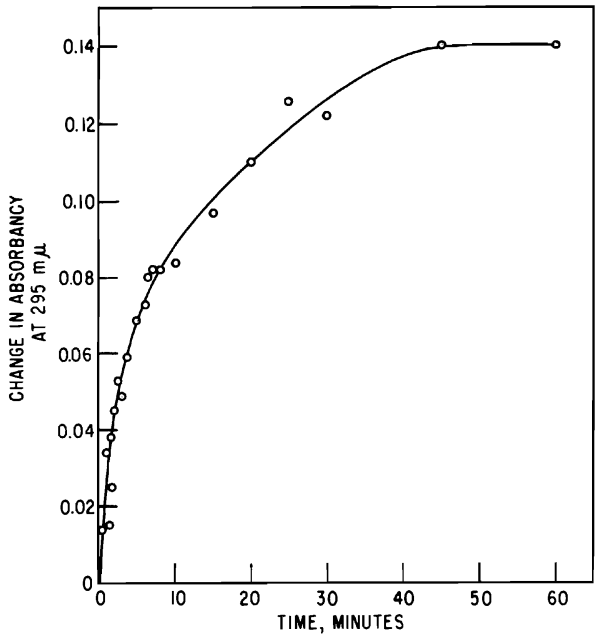

Fig. 15. Time dependence of the ultraviolet absorption of papain at 295 mμ at pH 12.3 and 27°. Papain concentration: 1.8×10^{-5}M. Light path: 1 cm.

very similar to that observed with papain (155).

The slow change in absorbancy observed with bovine serum albumin at pH values greater than 12, on the other hand, shows the greatest change at 265 mμ, a smaller one at 278 mμ and the smallest at 295 mμ, and has thus been attributed to a denaturation process (114).

Change in Molar Extinction.

The molar extinction at 295 mμ of all the solutions of papain in the pH range 5-8 is 12,500. This value was taken as the molar extinction of papain before the ionization of the phenolic hydroxyl groups. When the absorbancy at 295 mμ vs. time curves at pH 12.2, pH 13, and pH 14 are examined (e.g., Fig. 15), it is clear that all these approach the limiting value of 52,800. Amino acid analyses (4) as well as spectrophotometric determinations (see below) have indicated the presence of 17 tyrosine residues in the papain molecule. The total change in the molar extinction at 295 mμ is 40,300. This corresponds to a change of 2370 per tyrosine residue. This is in good agreement with the value of 2300 for tyrosine at the same wavelength (114). Tanford and Roberts (114) found the change at 295 mμ per phenolic hydroxyl residue to be 2430 in the case of bovine serum albumin. The value of 2630 was obtained in the case of ribonuclease (155).

The $\Delta\epsilon_{res.}$ (at 295 mμ) value of 2370 may be used to calculate the number of phenolic hydroxyl groups ionized at any pH. The results of such a calculation are summarized in Table XIV.

Reversibility.

The titration curve shown in Figure 16 can be fitted with a single S-shaped curve in the pH range 8.0-11.5. Over this pH range, the ionization is completely reversible. Exposure to pH values higher than 12.0 leads to irreversible changes in the ionization behavior. The curve obtained by

back-titration of a solution which had been left at pH 13 for a sufficient time for the attainment of ionization equilibrium is shown in Figure 16.

After exposure to pH greater than 12 for a few minutes, the protein becomes insoluble at pH values below 9.5, (presumably, because of denaturation and aggregation), and the ionization reversal curve cannot be continued below this pH. The results obtained in the pH range 9.5-13.5 indicate that all the 17 tyrosine -OH groups show normal ionization behavior after the exposure to pH values greater than 12 for sufficient time to attain ionization equilibrium.

Spectrophotometric Determination of the Tyrosine-Tryptophan Content of Papain.

The ultraviolet absorption spectrum of papain in alkaline solution was studied under a variety of conditions. The methods of Goodwin and Morton (193) and of Benche and Schmid (194) were applied to the determination of the tyrosine-tryptophan ratio in papain under the various conditions used. In agreement with the results obtained by measuring the $\Delta\epsilon_M$ at 295 mμ, it was found that the true tyrosine to tryptophan ratio of 3.4:1 (indicated by amino acid analysis) was approached only after prolonged exposure of the protein to alkali (see Table XV for details). After 24 hours at pH 13 and 27°, or, 3 hours at pH 13.5 and 39°, a ratio of 3.25:1 was obtained (Figure 17).

Since the quantity of papain in the solutions studied was known, it was possible to calculate the number of moles of tyrosine and tryptophan per mole of papain. It was found that the amount of tyrosine was 16.7 ± 0.5 residues per mole and the amount of tryptophan 4.8 ± 0.1 residues per mole. These values are in excellent agreement with earlier studies, which had shown the presence of 17 tyrosine and 5 tryptophan residues in papain (4).

TABLE XIV

IONIZATION OF PHENOLIC HYDROXYL RESIDUES IN PAPAIN

pH	Conditions	No. of ionized phenolic hydroxyl groups
11.8	5 min. at 27°	11.6
13.0	30 sec. at 27°	15.5
14.0	2 hours at 39°	17.0

All solutions 0.2M in NaCl.

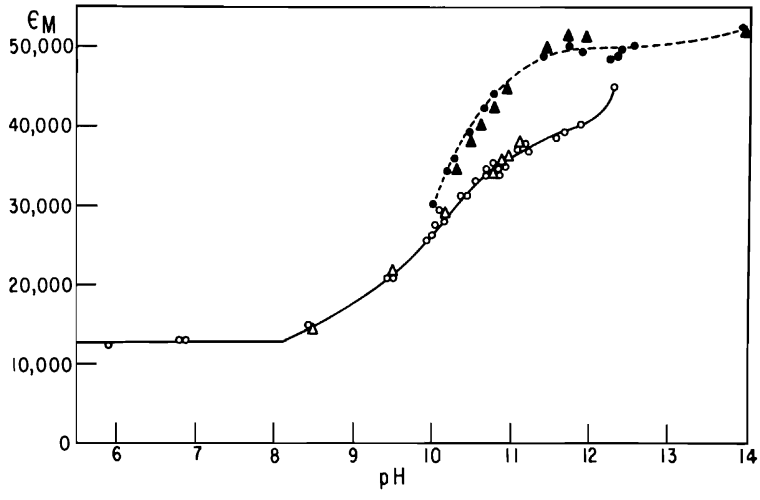

Fig. 16. Ionization of the phenolic groups in papain.

Direct titration: ○ ionic strength 0.01.

△ ionic strength 0.20.

Reversed titration: Reversed after exposure to pH 13 for sufficient time to attain complete ionization equilibrium. ● ionic strength 0.01, ▲ ionic strength 0.20.

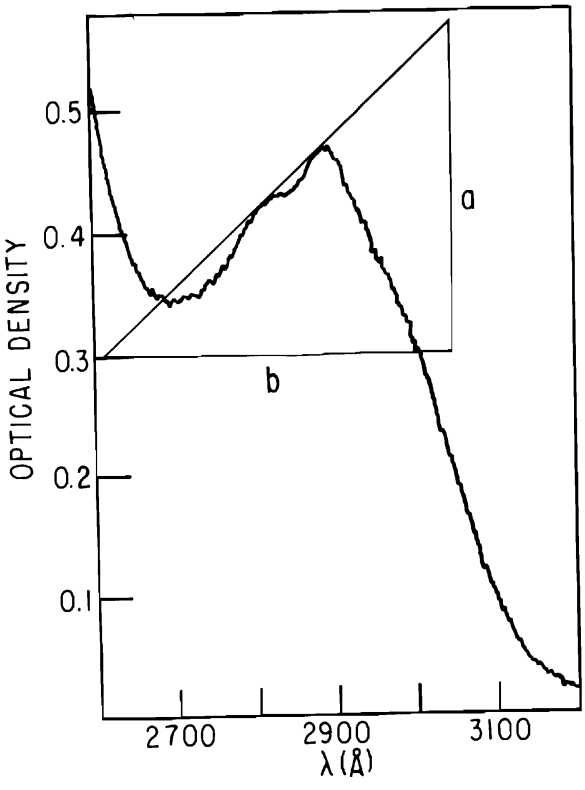

Fig. 17. Determination of the tyrosine-tryptophan ratio in papain by the method of Schmid and Benche.

$$S = \frac{(a/b)\ 10^3}{A_{max.}} = \frac{\Delta A.10^3/\Delta m\mu}{A_{max.}} = +13.3$$

Tyrosine/Tryptophan = 3.25/1

Spectrum recorded after the protein had been exposed to 0.1 N NaOH for 24 hours at 27°.

Perlmann (195) reported that prolonged exposure of ovalbumin to alkali led to a destruction of some tyrosine residues. The importance of this effect was evaluated for papain and ovalbumin under the conditions used above for determination of the tyrosine-tryptophan ratio. The results are listed in Table XVI.

The loss of tyrosine, as determined by the decrease in absorption at 295 mμ, was found to be approximately 3 per cent in the case of ovalbumin and 4 per cent in the case of papain, after a 17-hour exposure to 0.1N sodium hydroxide at 27°.

This observation correlates well with the observed tyrosine-tryptophan ratio of 3.25:1 after this treatment. This ratio gives a value of 16.25 tyrosine residues per mole of papain (assuming no tryptophan loss) as compared with 17 residues indicated by amino acid analysis. The loss of tyrosine indicated by the results in Table XVI is 0.68 residue.

The determination of the tyrosine-tryptophan ratio in ovalbumin gave 2.60:1 by the Benche and Schmid method (194) and 2.80:1 by the Morton and Goodwin method (193) (see Table XV), in good agreement with the value of 2.5:1 obtained by Benche and Schmid (194).

In the case of lysozyme, the reported tyrosine-tryptophan ratios of 0.46:1 (Benche and Schmid, (194)) and 0.6:1 (Goodwin and Morton, (193)) were approached only after prolonged exposure to alkali. (See Table XV).

Denaturation of papain, ovalbumin, or lysozyme by heating at 92°, at pH 1.7, for 5 min, prior to adjusting the solutions to pH 13, had only a slight influence on the tyrosine-tryptophan ratio in each case (see Table XV).

TABLE XV

TYROSINE-TRYPTOPHAN RATIO IN PAPAIN, OVALBUMIN, AND LYSOZYME AS DETERMINED SPECTROPHOTOMETRICALLY UNDER VARIOUS CONDITIONS

Protein	Conditions	No. of dets.	Tyrosine/Tryptophan*	
			Benche and Schmid (194)	Goodwin and Morton (193
Papain	pH 13, 27°, 5 min.	4	2.70±0.10	2.50±0.20
	pH 13, 27°, 17 hrs.	4	3.20±0.05	3.15±0.02
	pH 13.5, 39°, 90 min.	2	2.95±0.05	2.87±0.08
	pH 13.5, 39°, 180 min.	3	3.25±0.15	3.25±0.20
	Denatured at pH 1.7 at 92° for 5 min. then pH 13.5, 39°, 30 min.	2	2.80±0.10	2.84±0.17
Lysozyme	pH 13, 27°, 5 min.	4	0.28±0.02	0.47±0.02
	pH 13, 27°, 17 hrs.	2	0.49±0.02	0.67±0.02
	Denatured at pH 1.7 at 92° for 5 min., then pH 13, 27°, 5 min.	2	0.32±0.01	0.51±0.02
Ovalbumin	pH 13, 27°, 5 min.	2	2.65±0.05	2.85±0.20
	pH 13, 27°, 17 hrs.	2	2.60±0.05	2.80±0.10
	Denatured at pH 1.7 at 92° for 5 min., then pH 13, 27°, 5 min.	2	2.65±0.10	2.85±0.20

* Tyrosine/Tryptophan Ratios Reported in the Literature.

		Reference
Papain	3.4:1	Kimmel and Smith (4)
Lysozyme	0.46:1	Benche and Schmid (194)
	0.6:1	Goodwin and Morton (193)
Ovalbumin	2.5:1	Benche and Schmid (194)
	2.5:1	Crammer and Neuberger (153)

TABLE XVI

ABSORBANCY OF OVALBUMIN AND PAPAIN 5 MINUTES AND 24 HOURS AFTER EXPOSURE TO 0.1M SODIUM HYDROXIDE AT 27°.

λ(mμ)	Ovalbumin		Papain	
	5 min.	24 hours	5 min.	24 hours
270	0.295	0.300	0.390	0.345
275	0.298	0.301	0.395	0.360
280	0.335	0.339	0.435	0.410
285	0.360	0.360	0.455	0.434
290	0.387	0.382	0.482	0.469
295	0.331	0.321	0.408	0.392
300	0.253	0.251	0.323	0.301
305	0.172	0.161	0.210	0.203
310	0.090	0.080	0.100	0.100

Protein concentration: Ovalbumin 0.24 mg./ml.

Papain 0.164 mg./ml.

DISCUSSION AND CONCLUSIONS

The spectrophotometric titration of the phenolic hydroxyl groups in papain indicates that all of these groups in the papain molecule show abnormal ionization behavior. Eleven or twelve of these groups have a pK of approximately 10.2-10.4 (as compared to 9.55 for tyrosine itself) and ionize similarly to those of bovine serum albumin (114). Three to four groups ionize slowly at pH values between 12 and 13. This behavior is analogous to that observed for three of the six phenolic -OH groups in ribonuclease. Above pH 13, a slow ionization of 1-2 phenolic -OH groups takes place. These two groups show a unique ionization behavior. In ovalbumin, eight of the ten tyrosine hydroxyl groups show abnormal ionization behavior (153). All of these, however, ionize immediately on exposure of the protein to pH 13 at room temperature. Only 15 of the 17 tyrosine -OH groups of papain ionize immediately under these conditions. The remaining two groups ionize slowly.

These data emphasize the extreme tightness of at least a part of the secondary structure in papain, already indicated by the denaturation studies of Hill, Schwartz and Smith (144).

As mentioned in the General Introduction, extensive degradation of papain with leucine aminopeptidase results in the formation of an active fragment of papain containing 76 residues (10). As this fragment contains only 4 tyrosine residues (10), it would appear that 13 of the 17 tyrosine residues in papain are not involved in maintaining the integrity of the secondary structure in the vicinity of the active site. This conclusion is consistent with the observation made above that the ionization of 11-12 tyrosine residues is reversible. The reversible ionization of phenolic hydroxyl residues in proteins has been taken generally to indicate that these residues

are not involved in bonds important for maintaining secondary structure (114).

It has been shown that many proteins are not susceptible to hydrolysis by leucine aminopeptidase unless they have been denatured (197). It is possible, therefore, that the active fragment owes some of its resistance to further breakdown by the aminopeptidase to bonds involving the side-chains of the four tyrosine residues within it. Unfortunately, no information is available on the ionization behavior of these residues.

The tyrosine-tryptophan ratio obtained by spectrophotometric means agrees well with that obtained by amino acid analysis.

CONCLUDING DISCUSSION

In the General Introduction to this Thesis, it was pointed out that the work of previous investigators posed two major problems. The first of these is that of the two "missing" sulfur atoms, and the second, that of the chemical nature of the "active site" of papain.

The results of the present investigation provide the answer to the first of these two problems.

The investigation of the sulfhydryl content of native, unactivated papain by a number of methods reveals only a fraction of a mole of sulfhydryl per mole of protein.

The disulfide interchange reaction clearly indicates the presence of eight groups in papain which, after exposure to 9.6N HCl at 39°, behave in a manner analogous to the cystine and cysteine residues in other proteins. In fact, it appears to be reasonable to assume that under the conditions of the interchange reaction, we are dealing with eight half-cystine residues.

The determination of the disulfide content of papain by argentimetric amperometric titration with sulfite as the cleaving agent clearly shows the presence of three disulfide bonds in papain at near-neutral pH, whether in the native, urea-denatured, or detergent denatured protein. When papain is exposed to pH 1.7 for a short period of time and then titrated at near-neutral pH in 8M urea, four disulfide bonds may be titrated with silver ion in presence of sulfite.

Finally, determination of cysteic acid in acid hydrolysates of papain oxidized with performic acid, clearly shows the presence of eight moles of cysteic acid per mole of papain.

The above evidence indicates that after acid treatment or oxidation (which is also performed under acidic conditions), the sulfur content of papain can be accounted for completely on the basis of the half-cystine content of the protein.

The answer to the first of the two problems posed above unfortunately has not automatically provided an answer to the second.

The studies on the thiol content of active papain clearly indicate that the activation of papain is associated with the appearance of one thiol group per mole of protein.

The hypothesis that activation of papain involves the cleavage of a disulfide bond is subject to a number of serious criticisms.

1. A reductive cleavage of an intramolecular disulfide by column reduction should lead to the appearance of two thiol groups. Actually, Finkle and Smith (15) have shown that only one such group appears as a result of this treatment. This point has been confirmed in the present study.

2. The assumption of an inter-molecular disulfide bond requires the concomitant assumption that inactive papain is a dimer. No evidence for the existence of such a dimer has been found (3).

3. The fact that acid treatment is required before the fourth disulfide bond can be titrated with silver ion in the presence of sulfite is plausibly explained on the basis of the simple assumption that the denaturation resulting from this treatment renders the bond accessible to sulfite, or changes its environment. The finding that 8M urea at 37° does not bring this about argues against this explanation.

The above considerations indicate that while papain undoubtedly does have three disulfide bonds similar to those found in other proteins, the question of the fourth bond is not so clearly resolved.

In 1958, Smith (22) has suggested that inactive papain exists in an oxidized form, corresponding to the "sulfenic" state. Activation could then be considered as representing reduction to the thiol.

This suggestion has received support from the results obtained with the streptococcal proteinase (196). This enzyme contains only one half-cystine residue per mole. In analogy with papain, the streptococcal proteinase requires activation by thiol compounds. No sulfhydryl can be detected in the inactive enzyme. This situation is clearly best explained by assuming that in the inactive enzyme the sulfhydryl has been oxidized to the -SOH form and that activation consists in the reduction to the sulfhydryl.

If we assume that inactive papain does have a sulfenic acid group, the remaining problem is that of its partner - the eighth sulfur atom. It is possible that the latter is present in an acid-labile covalent linkage, R-S-X, between the half-cystine and an ϵ-amino group of lysine, an anhydride linkage with a γ-COOH or a β-COOH of a glutamic or aspartic acid residue, or an ester linkage between a sulfenic acid and a phenolic hydroxyl group of tyrosine or the -OH group of a serine or threonine residue.

The appearance of the fourth disulfide bond after acid treatment could then be interpreted as follows:

$$\text{R-S-X} + \text{H}^+ \longrightarrow \text{RSH} + \text{X}^+ \qquad (1)$$

$$\text{RSH} + \text{RSOH} \rightleftharpoons \text{RSSR} \qquad (2)$$

where R-SOH is the oxidized form of the sulfhydryl involved in the active site of papain.

The study of the region of sulfur absorption of proteins in the ultraviolet region, which may have led to the detection of unusual sulfur linkages is unfortunately greatly complicated by a variety of effects due to other groupings under the conditions necessary for cleavage of acid-labile linkages.

The studies on partial acid hydrolysates of oxidized papain have indicated that a cysteic-cysteic sequence is probably present (69). Further, the studies on tryptic and chymotryptic hydrolysates of papain, S-carboxymethyl papain and oxidized papain indicate that there is a high concentration of half-cystine residues in some portion of the molecule.

A large number of reactions, e.g., dismutations, or reactions such as reaction 2 shown above, which depend on steric factors, could be visualized as being favored by the proximity of the half-cystine residues resulting both from sequential arrangement and the folding of the peptide chain.

The ultimate answer to the problem of the chemical nature of the "active site" in papain will have to await the determination of the complete amino acid sequence of this protein. The development of specific methods for the demonstration of highly labile linkages in proteins may also be necessary.

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